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STUDIES ON THE EFFECTS OF WHOLE-BODY GAMMA RADIATION ON CHOLESTEROL METABOLISM

by

DONALD M. LYSTER

A THESIS
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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled: "STUDIES ON THE EFFECTS OF WHOLE-BODY GAMMA RADIATION ON CHOLESTEROL METABOLISM", submitted by Donald M. Lyster, in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The effects of various doses of whole-body gamma radiation on cholesterol metabolism were studied in different organs of the rat. Both free and esterified levels of cholesterol were observed. The pool size of each was determined spectrophotometrically and the rate of synthesis evaluated after injection of ^{14}C-acetate. The effect of radiation on coenzyme A availability was estimated *in vitro* by measuring the degree of acetylation of sulfisoxazole in the rat liver.

There was an initial post-irradiation increase in the amount of free cholesterol and a decrease in the amount of esterified sterol. This trend was evident for a longer period of time in the kidneys when compared to the liver. After this phase, an increase in the esterified cholesterol pool and its rate of synthesis was apparent. The esterified cholesterol pool was replenished by day 20 at all irradiation dosage levels while the free cholesterol pool was not normal by this time. It would seem from the results of this investigation that irradiation did not have a direct effect on the synthesis of cholesterol, but that the rate of synthesis was determined by the size of the pools.

It is proposed that irradiation stimulates a mobilization of free cholesterol, a portion of which may have been derived from the esterified cholesterol fraction. The kidneys may be acting as a reserve supply or buffer organ for

this steroid as increased amounts of the free sterol are noted for longer periods of time in this organ. Increased amounts of cholesterol are now available and these may be transported to the adrenal for the synthesis of other steroid hormones.

Although the lower irradiation doses showed an effect on the ability of the liver to acetylate sulfisoxazole, no correlation between this and cholesterol synthesis could be established.

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INTRODUCTION

Although many studies concerning the effects of ionizing radiation on cholesterol metabolism were reported, a number of conflicting results are apparent in the literature. However, it is generally accepted that there is a stimulation of cholesterol production but it is uncertain as to the actual cause, be it direct or indirect. Gould *et al.* (1) suggested that increased use of cholesterol decreases the amount present in the plasma and this decrease causes a stimulation in synthesis. Hanel *et al.* (2) also agree that an increase in synthesis may be due to a decrease in the pool size but they observed an initial drop after exposure to 400R whole-body dose and attributed this to the direct effect of irradiation. Nims and Sutton (3) suggest that the changes noted for cholesterol may be due to stimulation of the pituitary-adrenal system. What happens to this rapidly synthesized cholesterol is not clear, but it must be rapidly used since the total cholesterol content of liver, intestine, spleen and carcass changes little (4). It may also be that animals, in their struggle for survival during post-irradiation stress, require and mobilize additional fat and synthesize more steroids.

It is also noted that acetyl-CoA occupies a prominent position in the metabolic route of many metabolites in the animal organism during its normal function. However,

many reactions where acetyl-CoA is involved are disturbed after whole-body irradiation such as fatty acid and cholesterol synthesis. Some investigators have also shown a reduction in the ability of the liver to acetylate sulfanilamide after irradiation (5,6) which may indicate a disturbance in the molecule or its function.

It has also become apparent that there may be a difference in dose-effect of irradiation depending on the species, size and sex of the animal and also conditions under which irradiation took place. Because of this it is difficult to compare the results of various investigators unless all conditions are identical.

With these thoughts in mind, it was decided to investigate the effects of various doses of gamma irradiation on cholesterol metabolism and sulfisoxazole acetylation. Whole-body exposures of 50R, 400R and 700R of gamma rays were chosen to represent the various effects of radiation from a low to a lethal dose. Analysis of the results on days 1, 3, 7, 11 and 20 post-irradiation were selected to correspond to the three levels of response in the adrenal as postulated by Selye (7) and also to be practical within the conditions of our laboratory.

The proposed investigation was to provide the following information as to the various effects of gamma rays

on cholesterol metabolism and acetylation.

1. The effect of gamma rays on the coenzyme A in the liver as measured by the degree of acetylation of sulfisoxazole.

2. The degree of post-irradiation synthesis of free cholesterol in the liver, kidney and adrenals as measured by incorporation of acetate in the cholesterol molecule.

3. The levels of free cholesterol (pool plus synthesis) in the above three organs as measured by the determination of free cholesterol content.

4. The degree of post-irradiation cholesterol ester synthesis as measured by incorporation of acetate in the cholesterol moiety of the ester in the above three organs.

5. The levels of cholesterol (pool plus synthesis) in the above three organs as measured by the determination of esterified cholesterol content.

SURVEY OF THE LITERATURE

A. Biochemical Role of Acetyl-Coenzyme A

Acetyl-Coenzyme A (Acetyl-CoA) represents a focal point for sterol synthesis as well as acetylation in the body. Figure 1 represents the interrelationship between the coenzyme and the metabolism of fatty acids, cholesterol and ketone bodies.

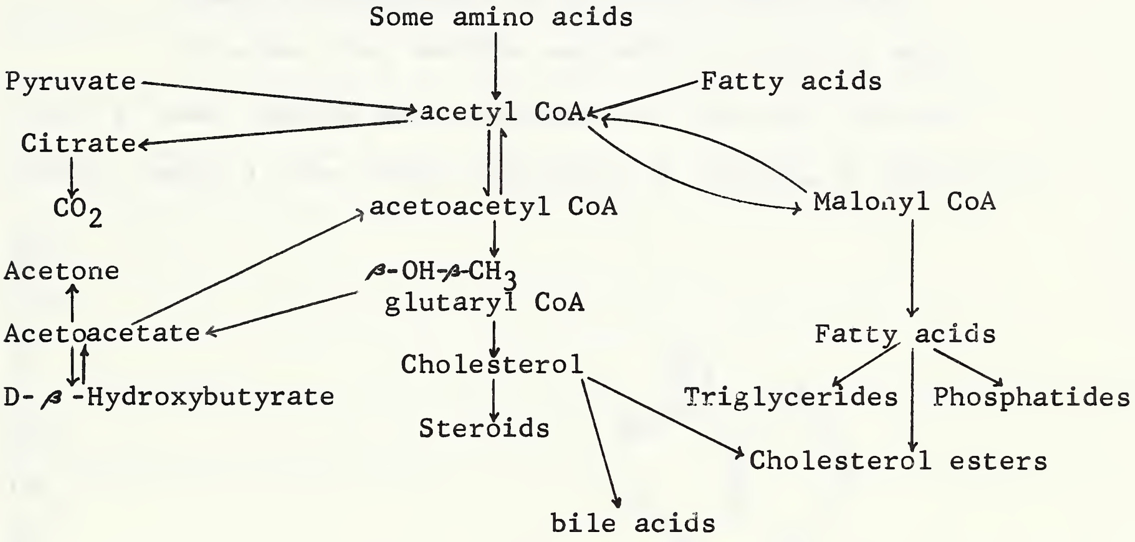

Figure 1

Role of Acetyl-Coenzyme A in Sterol Metabolism (8)

Acetyl-CoA is also required in the citric acid cycle in order that oxygen may be reduced and ATP be generated (Figure 2). For each revolution of the cycle, one mole of acetyl is consumed and converted into two moles of carbon dioxide. The net accomplishment of the cycle, per revolution, may be represented as follows:

$$CH_3COOH + 2O_2 \longrightarrow 2CO_2 + 2H_2O + \text{energy}.$$

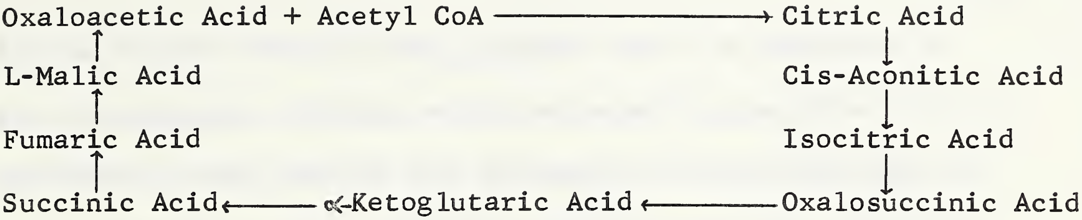

Figure 2

Major Intermediates in the Citric Acid Cycle.

In order that acetate may enter the citric acid cycle it must combine with coenzyme A to form the "active acetyl" which is the acetyl thioester of coenzyme A (Figure 3).

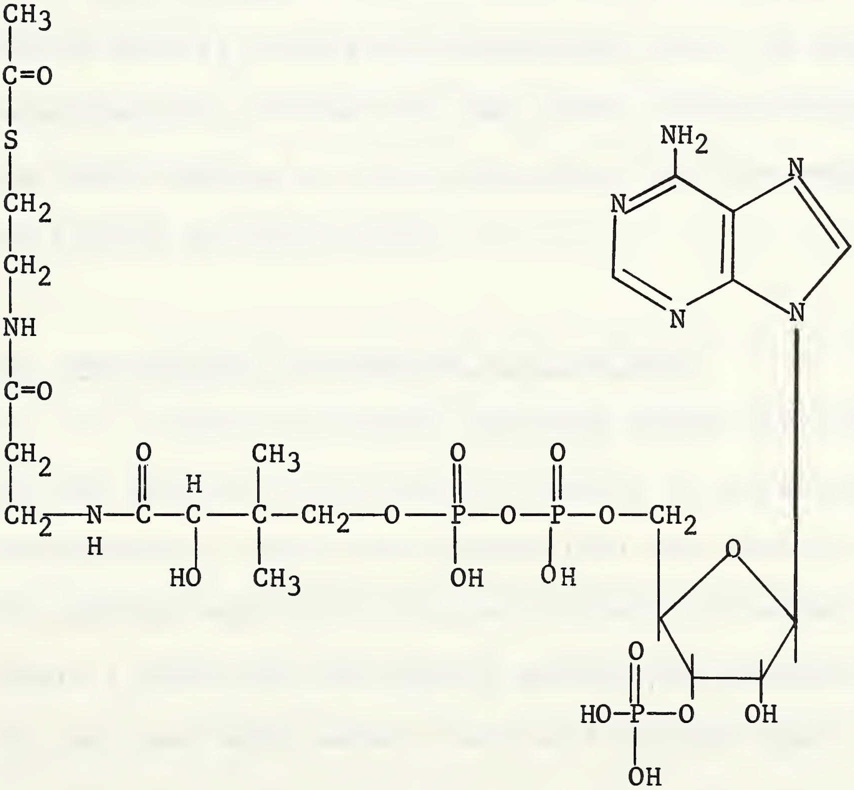

Figure 3

Acetyl-Coenzyme A (Acetyl-CoA)

In the "active acetyl" form, acetate can be a precursor in the biosynthesis of fatty acids and sterols, can give rise to acetoacetic acid, and is the biological acetylating agent in the synthesis of such compounds as acetylcholine, acetylsulfanilamide, etc. The activation of acetate to acetyl-CoA requires adenosine triphosphate (ATP) which in turn is converted to adenylic acid (AMP) and inorganic pyrophosphate (PP_1). Quantitatively, the synthesis of acetyl-CoA from

$$\text{ATP} + \text{acetate} + \text{CoA} \rightleftharpoons \text{AMP} + \text{PP}_1 + \text{acetyl-CoA}$$

acetic acid is relatively unimportant but it is used experimentally to study the many fates of acetyl-CoA. The two major sources of acetyl-CoA derive from the metabolism of glucose and fatty acids.

B. Distribution of Coenzyme A in the Body

Using a procedure employing pigeon liver extract and the Bratton and Marshall (9) method of sulfanilamide determination, Kaplan and Lipmann (10) were able to study the various amounts of Coenzyme A located in animal tissues. Table 1 shows that the highest amounts are generally found in the liver which seems to act as a storage organ.

Table 1

Coenzyme A Distribution in Animal Tissues [a].

	Human	Rabbit	Rat	Pigeon
Liver	-[b]	112	132	105
Adrenal	-	65	91	-
Adrenal demedullated	-	-	79	-
Kidney	-	50	74	-
Brain	-	40(cortex)	28	40
Heart	-	26	42	45
Testis	-	26	-	-
Intestine	-	-	26	-
Thymus	-	-	20	-
Skeletal muscle	-	6	-	-
Blood plasma	0	-	-	-
Red blood cells	3-4	-	-	-

[a] All values are given as units of coenzyme A per gram of fresh tissue.

[b] A blank - indicates no value given.

Generally, the liver was found to be the sole site of acetylation (11) and acetylsulfanilamide, once formed, was not decomposed in the animal body (12).

C. Mechanism of Action of the Acetylation Reaction

Klein and Harris (12), using rabbit liver slices, were able to demonstrate that the acetylating process seems to involve at least three factors. These are as follows: the stimulating effect of sulfonamide, the production of acetate which is usually the limiting factor, and the actual

coupling process. After a series of experiments, they suggested the following scheme for the acetylation of sulfanilamide by the rabbit liver:

$$\begin{tikzcd}
\text{Lactate} \arrow[r, "a"] & \text{pyruvate} \arrow[r, "b"] \arrow[d] & \text{acetate+sulfanilamide} \arrow[r, "c"] & \text{acetyl sulfanilamide} \\
& \text{acetaldehyde} \arrow[ur, "b"] & &
\end{tikzcd}$$

Reaction (a) is inhibited by anaerobiosis, thus limiting acetylation and reaction (b) represents an oxidative step. The conjugation of acetate and sulfanilamide in reaction (c) occurs only in the intact liver cell, is not reversible, is inhibited by high concentrations of iodoacetamide and arsenious oxide, is only slightly inhibited by anaerobiosis, and varies greatly in animals of the same species. The rate of acetylation remained constant although the concentration of sulfanilamide in the medium decreases which implies that the rate is dependant upon the original concentration. Lipmann (13) confirmed the results of Klein (12) using cell-free preparations. By means of enzyme preparations he demonstrated that in the case of sulfanilamide acetylation, the coupling with energy-yielding reactions depended on the supply of energy-rich phosphate bonds from adenyl pyrophosphate. In turn, adenyl pyrophosphate acted as an energy donor and was found to promote the conjugation of sulfanilamide. It was therefore postulated that the energy transfer between respiration

and acetylation occurs by way of phosphate bonds. The acetate and sulfanilamide are enzymatically induced to form a complex with adenyl pyrophosphate which exergonically breaks up into acetylsulfanilamide, adenylic acid, and inorganic phosphate.

D. Effects of Irradiation on the Acetylation Reaction

Some investigators have found that various doses of irradiation are not detrimental to the coenzyme A function and as such do not interfere with normal acetylation processes within the body (14-18). DuBois *et al.* (14) reported that when sulfanilamide was administered to irradiated and normal rats, the urinary excretion of free and acetylated sulfanilamide was not affected by either sublethal or lethal doses (400R, 600R, 800R, 1200R) of whole-body x-irradiation. It was concluded from this study that the *in vivo* functional activity of coenzyme A in the liver is not impaired by x-irradiation. The same investigation also showed that neither the rate, nor the extent of absorption and excretion of the drug was appreciably different in irradiated and in normal animals. Romantsev and Zhulanova (15) have demonstrated that when white rats were exposed to 650R of x-rays, the process of acetylating sulfanilamide does not undergo any change over the whole range of radiation sickness. Both in normal and irradiated animals, 60-85 per cent of the total amount of

sulfanilamide is excreted in the acetylated form. Upon exposure to a dose of 3320R the amount of free and acetylated sulfanilamide excreted in the urine in 24 hours was practically cut in half. The authors suggested this to be due to retarded absorption of the sulfanilamide after intraperitoneal injection. Thomson and Mikuta (16) exposed rats to 1000R and found that the animals retained their capacity to acetylate p-aminobenzoic acid and sulfanilamide for at least three days after exposure. Gardella and Lichtler (17) have examined the effects of irradiation on coenzyme A activity of pigeon liver *in vitro* and found it was not inactivated by as much as 12,000R. Similarly, *in vivo* irradiation with 1000-1600R did not measurably alter, within 8 hours, the pre-irradiation content of coenzyme A in rat liver, rat thymus, or Yoshida Sarcoma cells. Yam and DuBois (18) have studied the effects of 700R x-irradiation on an *in vitro* acetylation system in which coenzyme A was the rate limiting component of the reaction. Only transient changes in coenzyme A concentration were found, and the authors concluded that radiation does not exert any important action on coenzyme A.

On the other hand, many investigators have reported that ionizing radiation does have an adverse effect on acetylation and coenzyme (19-25). Filippova and Seits (19) found that irradiation does indeed exhibit an inhibitory

effect on coenzyme A and its function in acetylation. The authors found that radiation decreased the coenzyme A concentration in the liver as well as the activity of the protein component of the acetylation system. A correlation was found between the severity of the radiation syndrome and the degree of inhibition of the enzymatic component of this acetylating system. The second stage of acetylation, namely the transfer of the "active" acetate onto the respective acceptor was shown to be the one affected by irradiation and not the first stage which consists of acetate "activation". The authors thus concluded that irradiation affected both the enzymatic and coenzyme components of the catalytic acetylation system. Hagen *et al.* (20) found that the *in vivo* acetylation of sulfanilamide was reduced by 50 per cent after 450R or 750R total-body irradiation and that the reduction does not return to normal for at least 30 days. The authors point out, however, that the same effect is observed after a nonspecific stress of the animal (eg. cooling) and as such, the response is not a specific radiation effect. The work of Koch and Hagen (21) confirmed the results obtained by Hagen *et al.* (20). After irradiation with 450R, Hanel and Wilian-Ulrich (5) found that the capacity of the liver to acetylate sulfanilamide fell on the first day with a significant minimum on the third day post-irradiation.

The levels were restored to normal by the seventh day. The authors suggested that irradiation of the rat did not affect the molecule of coenzyme A but introduced a shift in the metabolic pathways of fatty acids and cholesterol. "Fatty liver" has been noted after irradiation (22,23) and Agostini _et_ _al_.(24) found that this condition can be produced by irradiating rats with 50R daily for 23 days. The latter authors noted also that the chronic irradiation caused a decrease in the liver coenzyme A content. It was thus postulated that both coenzyme A deficiency and major free fatty acids availability play a pathogenic role in the formation of fatty liver by x-rays. Similar results have been shown in mice (25,6). After the irradiation of white mice with 700R, Strubelt (25) found there was a significant decrease in coenzyme A from the second day post-irradiation until the death of the animal. Such a decrease was noted in the liver, kidney, intestine, brain and spleen. Berndt (6) compared the effects of irradiation (690R) in mice which were allowed food _ad libitum_ and those which were starved for 24 hours. He found that there was no modification of the acetyl coenzyme A content except in the two hours post-irradiation period in the group of mice which were allowed food. The starved group of animals demonstrated a primary increase followed by a decrease and a subsequent increase on the second

day. The mice then exhibited a continuous decrease in coenzyme A activity.

Radiation sensitivity of coenzyme A in wet and in dry yeast cells has been studied by Hutchinson et al. (26). Only seventy per cent of the catalyst's activity was found to be readily destroyed in the wet cell and the remaining thirty per cent was at least ten times more resistent. An explanation for this behavior was presented on the basis that thirty per cent of the coenzyme A was in surroundings in which the indirect effect of irradiation is greatly reduced because of lower water content. This conclusion was presented as the authors have demonstrated that coenzyme A is very insensitive to the direct effects of irradiation. It has also been shown (27) that coenzyme A is present in cells in two different states. In E. coli cells, the co-factor molecules are all in one state which is susceptible to attack from water radicals. In the resistant state, coenzyme A exists to the extent of thirty per cent in yeast and seventy per cent in peas. On the other hand, resistance to radiation is essentially exhibited in all mammalian heart and liver cells. It is noted that all cells containing resistant coenzyme A also contain mitochondria. Part of the coenzyme A in some of the cells is so resistant to the action of ionizing radiation that it indicates that sensitive groups on the molecule are not usually in direct contact with water (27).

E. Biosynthesis of Cholesterol

After extensive investigation, the various steps in cholesterol synthesis from acetate have been shown and these are indicated broadly in Figure 4 (28). It is shown, as follows, that: (1) both carbon atoms of acetic acid contribute to the sterol molecule; (2) cholesterol contains the two carbon atoms of the acid in a definite pattern; and (3) every carbon atom in the sterol can be assigned to either the methyl or the carboxyl carbon of acetic acid.

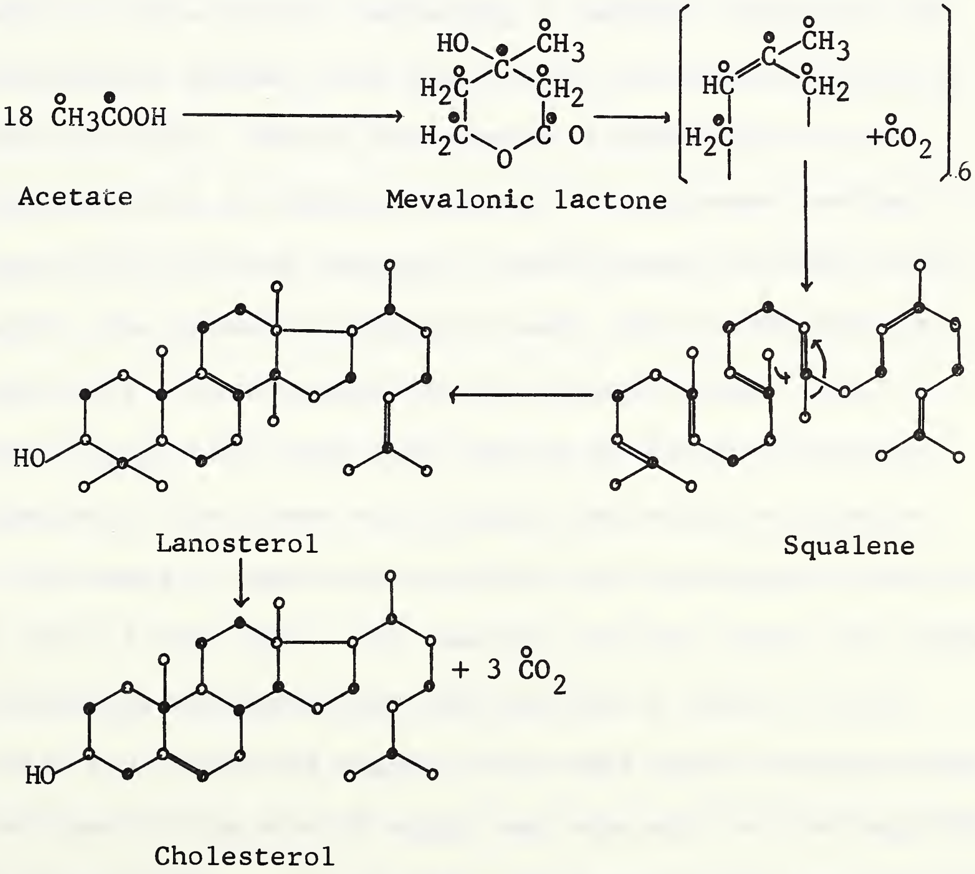

Figure 4

Overall Mechanism of Synthesis of Cholesterol from Acetate

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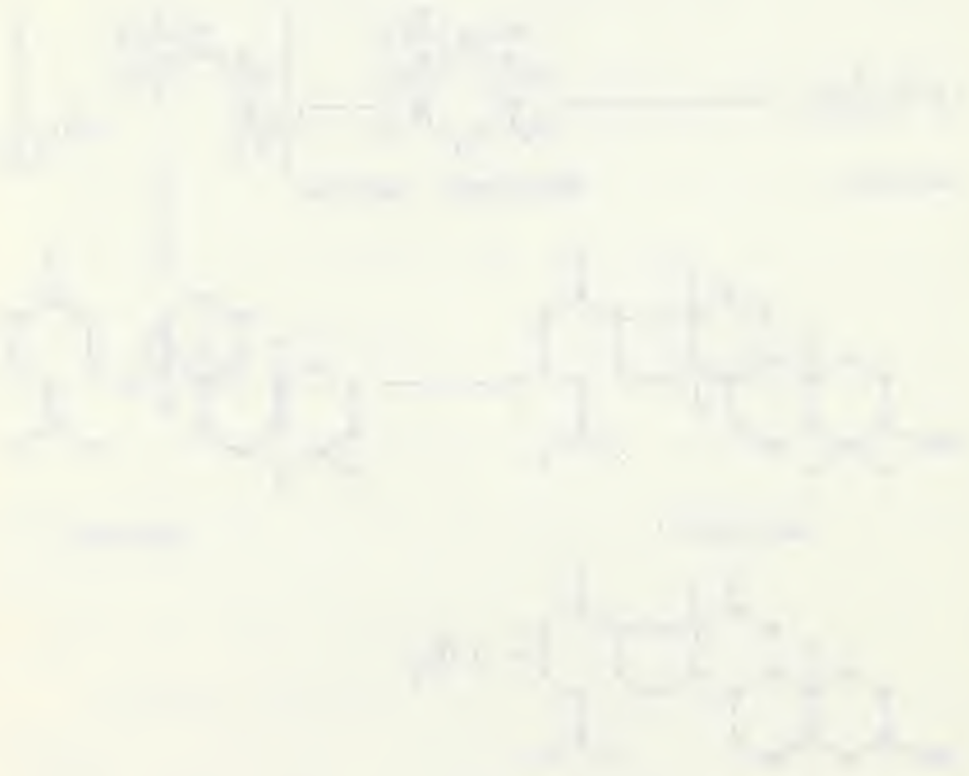

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Cholesterol, in all tissues other than nervous tissue, constitutes a "pool" which appears to exchange with serum cholesterol and this exchange is taking place continuously (29,30,31,32,33). Free cholesterol is shown to attain equilibrium in various tissues in a shorter period than do esterified cholesterol fractions (29). In the adrenal gland exists relatively small, rapidly turning over pools, and rather large, inert, reserve pools (33). The rate of cholesterol synthesis is markedly influenced by the available supply of this sterol, suggesting a feedback control of the biosynthetic pathway, and this control is exerted chiefly in the liver (34). When a disturbance of metabolism occurs resulting from an imbalance between the processes leading to deposition and those leading to mobilization of lipid in the depots, the dynamic biological steady state in the body is upset (35). In an experiment with hepatectomized dogs, Taylor _et al_. (34) found that labeled cholesterol molecules appeared in the plasma more rapidly than would be expected on the basis of the known turnover rate for plasma cholesterol of about 8 days (29). The specific activity values for plasma cholesterol were more than half as high as those of liver within four hours and suggest that under normal circumstances, the liver is the site of origin and also acts as the regulator of plasma cholesterol. A review by Glomset (36) also indicates

the liver to be the regulator of plasma cholesterol. However, cholesterol synthesized in the intestinal mucosa may appear in plasma by following the same pathway as dietary cholesterol. Tomkins and Chaikoff (37) have shown the nutritional state of the rat to significantly influence the capacity of the liver in synthesizing cholesterol from acetate. The above authors found that fasting for 24 hours or longer, or caloric restriction for several days reduced the ability of the liver to form cholesterol. It was also demonstrated that the administration of either glucose, a protein hydrolysate, or fat, restored cholesterol synthesis to normal levels in the fasted rat liver. The physiological regulation of the rate of cholesterol synthesis is concerned with one or more of the steps between acetate and mevalonic lactone, as there is increased incorporation of acetate into cholesterol after x-irradiation with 2400R, however no significant difference was observed in the conversion of mevalonic lactone to cholesterol (38). Bucher _et al._ (39) also found the rate-limiting step to be between acetate and mevalonic lactone.

F. Methods of Cholesterol Analysis

Cholesterol reacts with a variety of strongly acidic substances to give colored products. Based on this fact, many methods have been developed for its quantitative determination

and these may be generalized as follows: (a) methods for the determination of total cholesterol; (b) methods for the determination of free and ester cholesterol; and (c) methods of determination of total, free, and ester cholesterol. It is quite simple to analyze for total cholesterol (40), but the determination of free and esterified cholesterol is much more time consuming (41,42,43). A slight modification of the determination of total, free and ester cholesterol, using the reaction with ferric chloride and sulfuric acid, has also been employed (44,45,46). Webster (47) has observed the reactions of six cholesterol color reagents with hydrolyzed and unhydrolyzed petroleum-ether extracts of the same serum. He reported that the six color reagents were suitable for the estimation of cholesterol in the hydrolyzed extracts of serum. However, ferric chloride reagent and p-toluenesulfonic acid reagent were found to be the only suitable ones for the direct analysis of cholesterol esters, without preliminary hydrolysis. The iron reagent was considered the most satisfactory in view of its greater sensitivity and more stable color-complex.

G. Effects of Irradiation on the Liver

1. General Effects

Irradiation has many varied effects on the liver. It has been shown to prolong excretion time (48), increase liver weight (49), disturb the protein-forming function (50), cause abnormal mitotic figures (51), increase the formation of peroxide compounds (52,53), alter fatty acid synthesis (54,55) and disrupt the normal conjugation of steroids (56). Mehrotra (57) showed biochemical changes to be related to histopathological alterations in the rat liver after x-irradiation as they were significant at intervals when morphological changes were well pronounced. Histological studies of the exteriorised organ, exposed to x-radiation in doses of 3000 and 25,000R, showed that necrosis of the liver surface occurs in animals exposed to 25,000R, but deeper portions of the parenchyma did not show significant alterations. Agarwall *et al.*(58) also exposed rats to single doses of 3000 and 25,000R and investigated the livers at 1, 5 and 10 days post-irradiation. They also concluded that the rat liver is highly resistant to x-rays as only mild histologic and biochemical changes were noted in a few animals. The importance of the applied dose of x-rays has been stressed in a study by Stark *et al.*(59). Total-body irradiation of rats with 600R produced severe degenerative lesions in the

liver which were evident by the sixth or seventh day. A dose of 400R had no visible effects on liver and 500R produced only slight changes in some animals. They have also concluded that the hormones of the adrenal cortex are essential for the manifestation of the metabolic changes occurring during the physiological adaptation process resulting from stress.

2. Effects of Irradiation on Cholesterol Synthesis

Study of the effects of irradiation on the synthesis of cholesterol in the liver has been undertaken by many investigators and the general finding is that it is stimulated (60,61,62,1). Rats, mice and guinea pigs were subjected to whole-body x-irradiation and the uptake of carbon-14 from acetate -^{14}C into liver lipids was increased 20-fold in rats, 2-fold in guinea pigs and not at all in mice after 24 hours (60). Bartsch and Gerber (61) studied the incorporation of 1-^{14}C-acetate in perfused liver and believe that the increase usually observed in lipid content of the regenerating liver is due to changes in transport rather than to changes in synthesis. Perfusion of a normal liver with the blood obtained from irradiated rats also stimulated cholesterol synthesis (62), and this agreed with previous findings that irradiation of rats caused accumulation of lipid in the liver in the intact animal. Gould (1), found that whole-body

x-irradiation resulted in an increase in cholesterol biosynthesis in rat liver, a small increase in mouse liver, but no significant change in the livers of rabbits or guinea pigs. The increase in synthesis rate in the rat liver was proportional to dosage over the range 300 to 2400R and this increase in rate was about 100 per cent per 100R. Vinogradova (63) reported that the rate of renewal of cholesterol depended upon the dose of irradiation and also the phase of the radiation sickness. The cause of this increased rate of cholesterol synthesis was postulated by Gould (1) to be due to the decreased concentration of the cholesterol pool and, in the liver, a decrease of 0.12 mg per gm was correlated with a doubling of the synthetic rate. He has also shown that this increase in rate of synthesis is independant of the route of administration or amount of acetate given indicating a true change in synthetic rate. A comparative study was performed on non-irradiated adrenalectomized rats, whole-body x-irradiated rats, and a third group of animals which were adrenalectomized, then irradiated (64). It was found that incorporation of ^{14}C-acetate into cholesterol was lower in adrenalectomized animals, but synthesis was stimulated after irradiation. Guinea pigs irradiated with 600R exhibited a reduced amount of cholesterol and an intense restoration process in the liver on the first and third days post-irradiation

(65). An increase in cholesterol synthesis was also shown in dogs on day 3 after a single x-ray dose of 500R (66), but no perceptible change in synthesis was noted after 300R (67). Pande *et al.* (68) reported that irradiation-induced increases in hepatic synthesis of cholesterol could be due to an increased carbohydrate metabolism in the liver of irradiated, fasted animals. Thus, the fall in hepatic synthesis of cholesterol, which accompanies starvation, could be prevented by irradiation. When fasted irradiated and non-irradiated animals were force fed with glucose, it was found that the incorporation of acetate into cholesterol was almost identical in both groups and that this incorporation was higher than in control animals fed *ad libitum*. It was thus postulated that the decreases in the activities of the enzymes synthesizing cholesterol and the alterations in the regulatory factors which accompany starvation are prevented as a result of irradiation. The removal of this block on the conversion of acetate-1-^{14}C into fatty acids by x-irradiation is also demonstrated by using liver slice preparations (69). The effect was slight at 200R but increased progressively as the dose increased until at 2500R, the amount of fatty acids synthesized in the first 24 hours post-irradiation period was equivalent to that produced in normally fed rats. Tret'Yakova and Grodzenskii (70) found that the increased rate of cholesterol synthesis in organs

of old rats was basically not different from the rate in young rats with the exception of the testes.

3. Effects of Irradiation on the Cholesterol Pool

Angelico (71) and Kil'chevskaya (72) observed a decrease of liver cholesterol content after 42 and 72 hours post-irradiation. On the other hand, Elko and Di Luzio (73) noted that there was no alteration in the liver cholesterol concentration of rabbits 24 hours after 1000R of whole-body x-irradiation. Naqvi *et al.* (74) have also reported that no change in the amount of liver cholesterol was apparent four hours after an exposure to 800R or 900R, but 24 hours later the group exposed to 900R showed a decrease. It was also noted that 800R significantly reduced the esterified cholesterol concentrations. Coniglio *et al.* (75) found no significant change in total cholesterol from 2 to 42 days after irradiation that was directly attributable to irradiation. Shelley (76) has compared the effect of mixed neutron-gamma ray fields and x-irradiation on cholesterol levels in the rat liver. In the mixed field the highest levels were reached at 120 hours post-irradiation. X-radiation also produced increased cholesterol levels from 1 to 4 hours as well as 120 hours post-irradiation. Hanel and his co-workers (2) reported the cholesterol concentration in the liver fell 6 hours after

400R of x-irradiation, rose above normal on day one and then showed a continuous decline. By comparing the levels with the rate of synthesis they felt that the effect after 6 hours could be explained on the basis of change in synthesis but the initial drop must be due to a direct effect of irradiation. An investigation of the lipids of the mitochondrial membrane from rat liver indicated that cholesterol constitutes three per cent of the weight and that irradiation does not change this concentration (77). Therefore, whatever the mechanism is, whereby liver mitochondria are altered during an early period following irradiation, it does not involve major distribution of lipids in the mitochondrial membrane.

H. Effects of Irradiation on the Adrenal Glands

Many investigators have shown that the adrenals exhibit a biphasic response to the effects of ionizing radiation. In general, it has been demonstrated that the initial change occurs within the first few hours post-irradiation and the second change occurs between days three to seven (78-84). These are termed first and second reactions where the first reaction may be attributed to the effects of radiation and the second reaction is associated with bad general conditions such as loss of weight and diarrhoea (85). Various times for

analysis have been chosen and various doses of irradiation have been given.

1. First Reaction

At a dose of 650R whole-body irradiation, the adrenal corticosterone levels were increased at 2.5 hours post-irradiation and remained elevated for 48 hours. Irradiation of the head alone produced a rise in adrenal steroid levels at 72 hours with return to control levels by 24 hours. In hypophysectomized rats, no increase in corticosterone concentration in the adrenal was observed after x-irradiation, thus excluding a specific stimulating effect on the gland (78). However, when a dose of 700R was given, the *in vitro* level of aldosterone in the adrenal after three hours post-irradiation was higher than normal, but the corticosterone concentration was unchanged. After one day the aldosterone had returned to normal and the corticosterone rate was the same as in normal rats (79). When 1000R were administered, the initial phase lasted approximately 48 hours and the corticoid content of the adrenals indicated periods of arrested and increased adrenal cortex activity (81,82). When the effects of 1300R were investigated *in vitro*, essentially the reverse effect of irradiation on aldosterone and corticosterone production was found (80). The most significant

increase occurred with corticosterone and this effect was observed shortly post-irradiation. There was no distinct effect on aldosterone production. One investigator found an initial decrease in the content of corticosterone in the adrenal blood, but when ACTH was injected into the rat, within three hours after irradiation the amount of corticosterone in the blood reached control levels (84). It was concluded, therefore, that the reduced concentration of corticosterone in the adrenal blood was due to a disturbance of the pituitary regulatory function and not as a result of injury to the adrenals themselves. Shima and Matsuba (86) also found that the corticosteroid content of the blood, collected by direct cannulation of the adrenal vein of the rat, decreased about thirty minutes after whole-body or local (adrenal only exposed) x-irradiation at doses of 800R to 2000R. However, upon administration of ACTH to the irradiated rats, the corticosterone secretion was not raised to the normal level and it was suggested that x-irradiation has a dual action: systematic suppression of the adrenals, presumably via the hypothalamus-pituitary axis; and local inactivation of adrenal function itself. Corticosterone synthesis was seen to be elevated following doses of 600R (87) and 750R (88). The elevated biosynthesis of corticosterone and accelerated

incorporation of ^{14}C-acetate into free sterol was noticed at 24-48 hours post-irradiation with 600R.

Cholesterol, which is a precursor of the corticosteroids is also shown to exhibit this biphasic response to the effects of ionizing radiation (89,90). Three hours after 1500R total-body irradiation adrenal lipids showed a decrease in cholesterol esters which returned to near normal levels after 24 hours (89). Using 600R, a decrease of 50 to 70 per cent was noted in the first hours following irradiation, followed by an increase of 120 to 130 per cent beginning with the end of the first 24 hours, and this initial effect could not be modified with the administration to the rats of cysteamine prior to irradiation (90). Concentration of cholesterol is significantly decreased immediately post-irradiation following doses of 850R (91) and 900R (92). Shelley (76) has reported that exposure of rats to the LD100/30 of ionizing radiation produced by a mixed gamma-neutron field showed a significant post-irradiation decrease of cholesterol levels in adrenal glands. It is also noted that cholesterol response to the effects of x-rays is age dependent and Sladić et al. have shown that new born rats up to ten days of age do not react to this stress (93). A fall in concentration of cholesterol, three hours following x-irradiation, was only

observed for the first time in animals ten days old.

2. Second Reaction

After an apparent period of recovery, the animals once again exhibit a response due to the stress caused by the ionizing radiation, and they may or may not recover from this depending upon the severity of the dose. At 650R, the corticosterone levels in the adrenals showed a second increase at 48 hours post-irradiation, remained there for 48 hours followed by a decrease on days 5 and 7, and then a return to control levels by day 14 (78). With 750R (88) the *in vitro* synthesis of corticosterone showed an increase at 3 days post-irradiation and this increase continued until the death of the animal. The plasma level and adrenal synthesis rate of corticosterone were both highest in the terminal phase of acute radiation sickness. However, when the change in weight was considered, corticosterone production per unit weight of adrenal tissue was elevated 2-fold two hours after irradiation but later did not differ significantly from normal. This showed that the terminal hyperactivity was due to hypertrophy of the adrenal gland and suggested that the higher level of plasma corticoids after irradiation may result from their diminished catabolism by the injured liver, as well as from diminished secretion by the adrenals. With

1000R, the hormone content of the suprarenal was the largest shortly before death which occurred mostly on the seventh day after irradiation (82). Flemming and Yegin (81) also found this second increase to occur on day three after exposure to 1000R and that the activity of the adrenal cortex continues to increase until the death of the animal. After exposure to 1300R whole-body irradiation this second reaction took place by the second and third days and was marked by increased corticosterone production (80). A final prolonged decrease of cholesterol occurred about the sixth day and lasted from five to fifteen days (90). The administration of cysteamine to the rats prior to irradiation prevented this second decrease. The esterified cholesterol level showed a drastic reduction after returning to normal following 1500R whole-body irradiation (89). At the same time, increases in the levels of triglycerides and polyunsaturated fatty acids were observed.

3. Histological and Pathological Changes

Histological reports generally correspond to those obtained by means of biochemical methods. Mild degeneration and destructive changes in the glomerular layer cells were shown three hours after a single exposure to 300R irradiation and such changes were noted to return to normal after three to seven days (94). Graevskaya and Shchedrina (95), after

exposing mice to an x-ray dose of 600R, found an accumulation of lipids in the adrenal cortex mainly in the zona glomerulosa. Ishibashi (94) also showed more severe pathological changes with continuing increase in lipid granules at three to seven days after exposure to 600R. After exposure to 1200R, fine lipid granules appeared in the zona glomerulosa and zona fasiculata, and after seven days hardly any lipid granules were detected in the cortical layers. Using histochemical techniques, Brzezinski found that single doses of 700R enhanced the rate of biosynthesis of corticosteroids in rat adrenal cortex while 1200R decreased it (96). Single doses of 800R and 1000R elicited a primary response lasting from one to two days. The response was expressed in terms of mobilization of physiologically active substances. The secondary reaction, coinciding with the acute period of radiation sickness is characterized by a temporary reversible inhibition of corticoid synthesis followed by a compensatory rise in their formation. Borowicz (97) found that on the third and fifth days after irradiation, the reaction for ketosteroids occurred in the glomerular zone only, while it was absent in the fascicular area. However, in animals which were injected with ACTH,on the fifth day after irradiation the intensity and localization of the reaction were the same

as in healthy animals. It was thus concluded that lower hormonal activity of the adrenal cortex is a consequence of radiation injury to the pituitary and of the resulting ACTH deficiency. A correlation was found to exist between intensity of hormone secretion and the lipoid content of the adrenal cortex (98). A high hormone level in the blood after irradiation correlates with a low lipoid content of the adrenal cortex. The lowest level of blood hormone and the highest lipoid content of the adrenal cortex were observed 24 hours post-irradiation, and in the terminal phase there was a low lipoid content of the adrenal cortex and a high blood corticoid level. Exposure of rats to whole-body x-irradiation with a single dose of 1000R showed a decrease in the amount of lipid in the adrenal cortex after five hours (99). This change was first encountered in the zona reticularis, inner zona fasciculata, and then in the outer zona fasciculata. Three days later, the lipid nearly disappeared in the zona fasciculata and the zona reticularis, but in the zona glomerulosa no visible changes were seen.

I. Effects of Irradiation on the Kidney

Although the liver is the major organ of corticosteroid metabolism and secretion of steroids (100) the kidney is

also important of its conjugation and excretion (100,101).

Smith and Boss (102) have studied the renal function in rats whose exteriorized kidneys only were exposed to a single massive dose of x-rays to determine if any observed changes were the result of direct or indirect effects of irradiation. They concluded that both augmentation and depression of renal function were the result of the direct action of x-rays on the kidneys. Stevens and Berliner (103) found that the ability of the kidney to conjugate steroids *in vitro* is greatly reduced following 500R of whole-body irradiation. A slowdown in the elimination process was observed in all phases of radiation sickness by Zedgenidze (104) who concluded that functional disturbances were much more severe than the anatomical changes observed by histological examination. When the head, liver, adrenal, intestine, or hindquarters of the intact unanesthetized rat were irradiated with 3564 rep the changes in kidney weights roughly paralleled body weight loss (105). Distinct functional disturbances were observed when one or both kidneys received a single 1000R dose of irradiation (106). These were found to be reversible when only one kidney was irradiated but when both were irradiated, permanent damage could result. Following radiation doses of 1000R, 1500R, 2000R and 3000R to the site of the left kidney

in dogs, the irradiated kidney showed impaired function in all groups, and renograms of the kidney of the non-irradiated side also indicated impaired functioning (107). Morphological kidney examinations of whole-body irradiated swine have also shown degenerative changes and these changes were apparent for four to six months after irradiation with 800R to 1000R (108). Fischer (109) irradiated rats with 800R and found a drop of 36 per cent in kidney ascorbic acid and 42 per cent in cholesterol. When rats were anesthetized before or after irradiation, the decrease in cholesterol was almost completely eliminated. However, the decrease was less in rats anesthetized before irradiation. Naqvi (74) showed the cholesterol content of the kidney to be decreased 24 hours after irradiation with 900R and this was not prevented by administration of AET. (2-(2-aminoethyl)-2-thiopseudourea dihydrobromide).

EXPERIMENTAL METHODS AND MATERIALS

A. Selection of Animals

Male rats of the Sprague-Dawley strain were used throughout this investigation. Three to four week old animals, weighing 100 g on arrival, were allowed Purina Laboratory Chow and tap water *ad libitum* for four days prior to irradiation. The rats were subsequently deprived from food 12 hours prior to irradiation.

B. Irradiation Procedure

Randomly selected animals were irradiated with a Picker ^{137}Cs source. A 31.5 cm^2 applicator providing a 50 cm source-to-skin depth dose at 5 cm in our irradiation chamber was used. The irradiation cage consisted of a specially constructed Plexiglas container having the following dimensions: 38.5 cm x 30 cm x 10 cm. The cage contained 12 individual cylindrical compartments, positioned in 2 rows and each accomodating a single rat. The temperature of the entire set-up was controlled by means of circulating constant-temperature water maintained at 37^{o}. The air supply to each compartment was introduced by drilling uniformly-sized individual holes in the irradiation box. The air flow was adjusted to provide 2 liters of air per minute (as measured by a flow-meter) for each rat. Figures 5 and 6 demonstrate

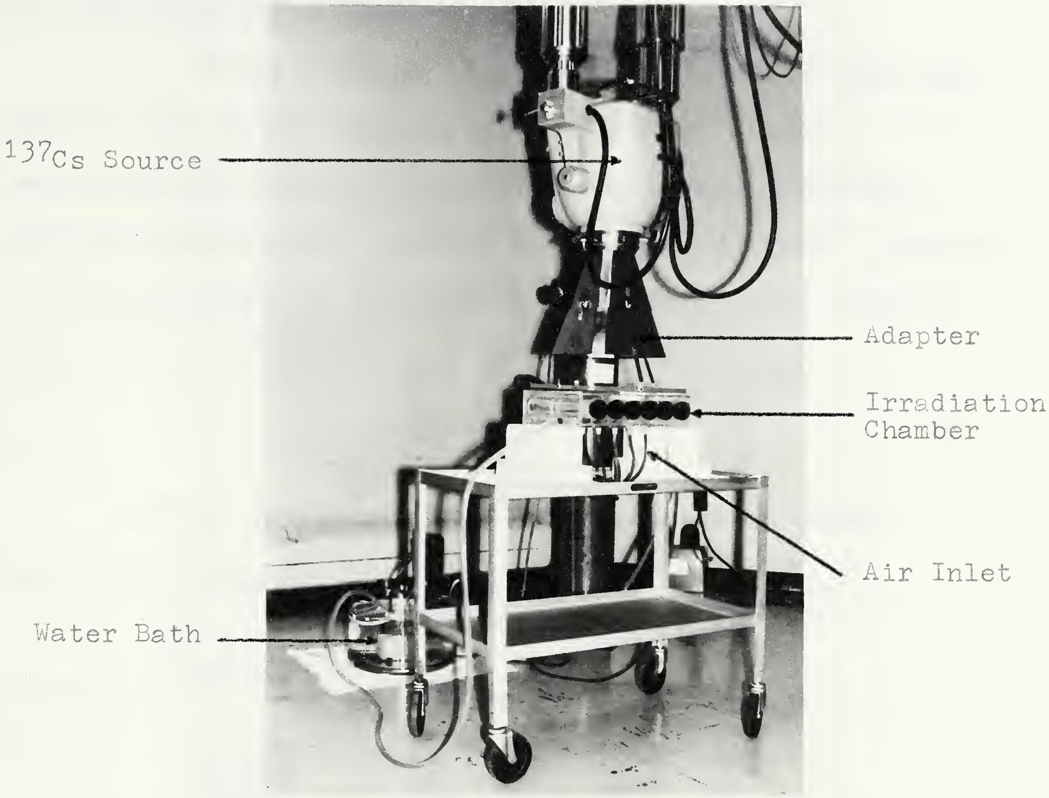

Figure 5 View of Irradiation Set-up

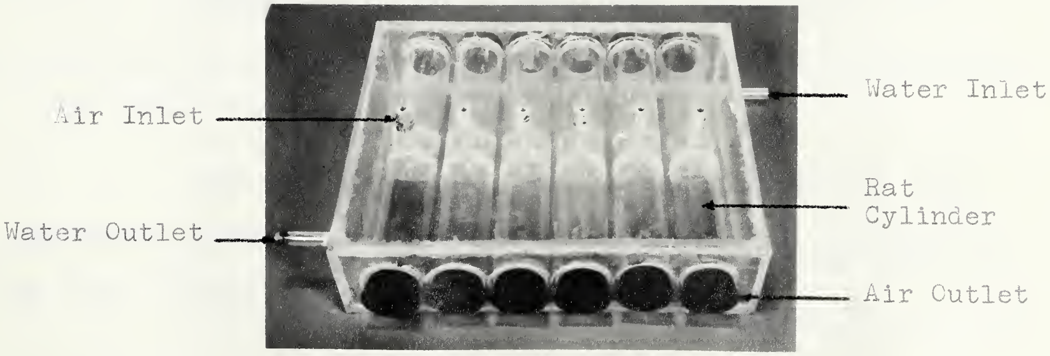

Figure 6 Bottom View of Irradiation Chamber

the irradiation arrangement used in this study.

Total exposure doses of 50R, 400R and 700R were administered at the average rate of 19R per minute. Measurement of the dose in the various compartments was effected by means of a Victoreen Condenser R-Meter[a]. Spatial dose distribution in the various compartments was found to be as given in Table 2.

Table 2

Spatial Dose Distribution in Irradiation Chamber*

		Compartments					
	Position	1	2	3	4	5	6
Row 1	caudal	17	18	18	18	17	15
	thorax	18	20	20	20	20	18
	head	20	24	24	24	24	20
Row 2	head	20	24	24	24	24	20
	thorax	18	20	20	20	20	17
	caudal	16	18	19	18	16	15

* All doses expressed in R/min.

The various animals were marked indicating their compartment number, placed in cages according to this marking, and then allowed food and water _ad libitum._

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[a] The Victoreen Instrument Co., Model 570, Cleveland, Ohio. R-Meter Chamber Model 154.

C. Preparation of Radioactive Solution

1-^{14}C-Acetate as the sodium salt (44.4 mCi/mM) was obtained from Nuclear-Chicago in vials containing 0.5 mCi. A stock solution was prepared from the latter by diluting with 5.0 ml of distilled water. A working solution was then prepared by diluting 1.0 ml of the stock solution to 5.0 ml with distilled water. The solution for injection thus contained 10 µCi of 1-^{14}C-acetate per 0.5 ml.

D. Injection of Animals

The effects of irradiation on the metabolism of cholesterol in rats was to be analyzed on days 1, 3, 7, 11 and 20 after exposure. Groups of six animals were selected for each experiment. The rats were randomly selected, one from each of six positions in a row in the irradiation chamber, and were grouped together in a separate cage. Food was withdrawn 24 hours prior to injection, but the animals were allowed to drink water *ad libitum*. The radioactive solution containing ^{14}C-sodium acetate was then injected intraperitoneally at a dose of 10 µCi per 100 g of animal weight. The rats were left for six hours in order to allow for cholesterol synthesis. A preliminary test had shown that optimal incorporation of acetate in cholesterol was after six hours from the time of injection.

E. Preparation of Tissues

Each rat was sacrificed by decapitation and blood drained out. The liver, kidneys, and adrenals were rapidly excised, cleaned from any adhering tissue, and then placed in a cold phosphate buffer solution consisting of the following: three parts of 0.04 M KH_2PO_4, seven parts of 0.04 M K_2HPO_4, 4 μM per ml of $MgCl_2.6H_2O$, 0.13 mM per ml of KCl, 10 μM per ml of glutathione, and five μM per ml of CH_3COOK. A portion of the liver, approximately 1 g in weight, was then immediately excised and used for the acetylation reaction. The remaining section, the kidneys, and adrenals were weighed accurately and frozen in dry ice before being stored at -10° for later use.

F. Biochemical Methods

1. In Vitro Procedures for Sulfisoxazole Analysis

a. Incubation Technique

The acetylation capacity of the liver was determined immediately using a modification of the procedure described by Hanel and Wilian-Ulrich (5). A piece of the liver was accurately weighed and homogenized in ice-cold phosphate buffer identical to the one used for tissue preparation. Each gram of liver tissue was homogenized with 1.5 ml of

buffer solution. A glass "Duall Tissue Grinder"[a] having a clearance of 0.004 in to 0.006 in was used and a 20-times repeated up and down motion effected the grinding. The glass rod was spinning clockwise at a speed of 400 rpm. Two milliliters of the homogenate were transferred to a 25 ml erlenmeyer flask containing 1.0 ml of 0.02 per cent (w/v) sulfisoxazole[b] in phosphate buffer. The preparation was incubated for 30 minutes at 37.5° with constant shaking at 70 cycles per minute in a Dubnoff Metabolic Shaking Incubator (Series 12-V-10)[c].

A mixture of gases consisting of 95 per cent oxygen and 5 per cent nitrogen was ~~being~~ introduced at the rate of 4 liters per minute. The acetylation reaction was interrupted by rapidly adding 1 ml of 12 per cent trichloracetic acid (TCA). The surplus of free sulfisoxazole was determined photometrically according to Bratton and Marshall (9) with slight modifications as described below.

b. Determination of Sulfisoxazole

To the solution in the erlenmeyer flask was added 30 ml of distilled water and after one minute 8 ml of TCA was added. The mixture was then filtered through Whatman No. 1

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[a] Kontes Glass Company, Vineland, New Jersey.

[b] Sold under the trade name of Gantrisin by Hoffmann-La Roche Ltd., St. Laurent, Montreal 9, Quebec.

[c] Precision Scientific Co., Chicago, Illinois.

filter paper. The free sulfisoxazole was determined in the filtrate as follows: One ml of a 0.1 per cent sodium nitrite solution in distilled water was added to 10.0 ml of the filtrate. After three minutes, 1.0 ml of a 0.5 per cent (w/v) ammonium sulfamate solution in distilled water was added and two minutes later 1.0 ml of a 0.1 per cent (w/v) solution of N-(1-napthyl)ethylenediamine dihydrochloride was mixed with the preparation. The absorbancy of the color formed was determined after 15 minutes by means of a Beckman DB Spectrophotometer[a] at a wavelength of 545 mμ. The amount of acetylated sulfisoxazole was calculated as the difference between the added amount and the remaining amount of free sulfisoxazole after the incubation.

A standard curve was determined by mixing 0.5, 1.0, 1.5 and 2.0 ml consecutively of the stock reagent (0.02 per cent (w/v) sulfisoxazole in distilled water) with 2 ml of a tissue homogenate prepared by homogenizing 1 g of liver with 1.5 ml of phosphate buffer. The reaction was stopped immediately by means of TCA (12 per cent) and extraction was performed as described before.

It was found that the readings for the standard curve were different from those standard solutions prepared without the addition of tissue homogenate. Table 3 demonstrates

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[a] Beckman Instruments Inc., Fullerton, California.

the difference in observed readings.

Table 3

Standard Curve for Sulfisoxazole

Without tissue homogenate		With tissue homogenate	
Amount added	Absorbance	Amount added	Absorbance
0.1 mg	0.32	0.1 mg	0.25
0.2 mg	0.60	0.2 mg	0.51
0.3 mg	0.85	0.3 mg	0.75
0.4 mg	1.10	0.4 mg	0.99

It was thus decided to use the method involving the addition of tissue homogenate for the preparation of a standard curve. An individual standard curve was determined for each experiment.

2. In Vivo Procedures for Cholesterol Analysis

a. Extraction of Total Lipids

The accurately weighed frozen livers, kidneys and adrenals were thawed, 2 ml of distilled water added, and the analysis carried out. Approximate weights for each of the above tissues were 2 g for the liver, 1 g for the kidneys and 0.05 g for the adrenals. The thawed organs were homogenized in a glass "Duall" tissue grinder at a speed of 400 rpm for 45 seconds (20 strokes). Each homogenate was then transferred quantitiatively to a 250 ml erlenmeyer flask containing 60 ml of a mixture of petroleum-ether[a] and

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[a] Matheson, Coleman, and Bell, bp. 60-90°, practical, Canadian Laboratory Supplies Limited, Edmonton, Alta.

95 per cent ethyl alcohol in the proportion of 1:3 respectively. The mixture was then heated in a boiling water bath for 1.5 minutes after which it was transferred to a 250 ml separatory funnel and allowed to cool to room temperature. The lipids and total cholesterol were then extracted four times using 25 ml portions of petroleum-ether (bp 60-90^{o}). It was found that the emulsion initially formed could be broken by the addition of a few drops of a saturated sodium chloride solution. Extracts of 100.0 ml of petroleum-ether were exactly obtained from each funnel. These were then evaporated in 250 ml boiling flasks using a flash evaporator[a] at a temperature of 35^{o}. The entire preparation was then reconstituted in 30.0 ml of low-boiling petroleum ether (bp 37.5-46.8^{o})[b]. Ten milliliters were removed for total cholesterol determination and the remaining twenty milliliters were used for esterified cholesterol determination. Each fraction was placed in a 40-ml centrifuge tube and evaporated to dryness at 40^{o}.

b. Separation of Total Cholesterol

Since some of the newly synthesized fatty acids

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[a] CaLab Model C Evaporator, Oakland, California.
[b] Fisher Scientific Company, Fair Lawn, New Jersey.

from 1-^{14}C-acetate are radioactive, the true activity, representing incorporation of acetate into cholesterol, can only be measured after the removal of such fatty acids by means of saponification. Thus, to the dry extract (from the 10.0 ml) was added 5.0 ml of previously prepared alcoholic KOH[a]. The tubes were stoppered, well shaken on a Vortex Jr. mixer[b] and incubated in a water bath at 37-40° for 55 minutes. They were then removed from the bath and, after cooling to room temperature, 10.0 ml of petroleum ether (bp 37.5-46.8°) were added and the tubes shaken vigorously for 30 seconds on the Vortex Jr. mixer. Five milliliters of distilled water were added and the mixture again shaken on the Vortex Jr. mixer for a period of 10 seconds. The tubes were centrifuged[c] at a speed of 1000 rpm for 10 minutes. Five milliliters of the petroleum-ether layer were removed, placed in a liquid scintillation vial[d], and evaporated for the determination of radioactivity. One ml of the same petroleum-ether extract was also evaporated in a 15 ml centrifuge tube at 40° for the spectrophotometric estimation of total cholesterol.

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[a] The alcoholic KOH was prepared immediately before use by adding 6 ml of 33 per cent KOH to 94 ml of 95 per cent alcohol.

[b] Scientific Industries Inc., New York, Model K-500-J.

[c] International Centrifuge, Model Cs, Boston, Massachusetts.

[d] Spectra 3, Nuclear Chicago Corp., Don Mills, Ontario.

c. Separation of Esterified Cholesterol

To each of the dry extracts, obtained after total lipid extraction, was added 1.0 ml of 95 per cent ethanol. The tubes were placed in a water bath at 80° for one minute. The tubes were then removed from the water bath and 1.0 ml of 1 per cent digitonin[a] was added in order to precipitate free cholesterol. The solution was shaken for 10 seconds and set aside for 10 minutes. Ten milliliters of petroleum-ether (bp 37.5-46.8°) was then added and the tubes shaken for 30 seconds. Five milliliters of distilled water were also added, the tubes again shaken for 10 seconds, and then centrifuged at 1000 rpm for 10 minutes. Five milliliters of the separated petroleum-ether layer, containing esterified cholesterol, were transferred to a clean 40-ml centrifuge tube and evaporated to dryness. Because of the possibility that some of the labelled fatty acids (biosynthesized from 1-^{14}C-acetate) may be present in the last extract, it was necessary to saponify and re-extract using the same procedure described under "separation of total cholesterol". The spectrophotometric analysis of the separated

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[a] 1 g of digitonin certified reagent, Fisher Scientific Co., Fair Lawn, N.J. was dissolved in 50 ml of absolute ethanol and diluted to 100 ml with distilled water.

cholesterol was performed using 2.0 ml of the final extract of petroleum-ether obtained after saponification. The radioactivity in 5 ml of the final extract was estimated by liquid scintillation counting.

3. Analytical Techniques

a. Liquid Scintillation Spectrometry

The petroleum-ether extracts containing ^{14}C-Cholesterol were placed in liquid scintillation vials and evaporated to dryness on a water bath. To each vial was added 15 ml of fluor consisting of 4 g of PPO[a] and 0.05 g of POPOP[b] per 1000 ml of toluene A.C.S. The samples were counted in an automatic Nuclear Chicago Liquid Scintillation Spectrometer[c] and the absolute disintegrations calculated by the channels ratio technique. A separate standard curve was calculated for each experiment using a series of Nuclear Chicago Carbon-14 quenched standards[d]. The activity in channel A was not allowed to vary by more than 1 per cent and the channel ratio was adjusted to 0.33.

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[a] 2,5-Diphenyloxazole, scintillation grade, Kent Chemicals Ltd., Vancouver, B.C.

[b] 1,4-Di-(2-(5-phenyloxazolyl)) -benzene, scintillation grade, Kent Chemicals Ltd., Vancouver, B.C.

[c] Nuclear Chicago Co., 720 Series, Des Plaines, Illinois.

[d] Model 180060, Liquid Scintillation ^{14}C-Quenched Standards Set, Nuclear Chicago Corp., Don Mills, Ontario.

b. Spectrophotometric Determination of Cholesterol

The procedure used for the spectrophotometric analysis of cholesterol was derived from that used by Webster (44) with slight modifications as described below. To the dried extract, obtained after separation of total or esterified cholesterol, was added 6.0 ml of working color reagent consisting of 2.0 ml of stock reagent diluted to 100.0 ml with glacial acetic acid. The stock color reagent was prepared by dissolving 5.0 g $FeCl_3 \cdot 6H_2O$ in 100.0 ml of glacial acetic acid. A blank was prepared by transferring 6.0 ml of the working color reagent to a clean, dry, 15 ml centrifuge tube and both tubes were placed in a water bath at 80^o for 5 minutes to dissolve the cholesterol residues. The tubes were then removed from the water bath, allowed to cool at room temperature, and 4.5 ml of concentrated sulfuric acid added carefully onto the side of the inclined tube thus forming two distinct layers. The two layers were intermixed by inverting the stoppered tube 10 times. The preparations were allowed to stand at room temperature for 30 minutes before reading the optical density with a Beckman DB Spectrophotometer at 560 mµ. The color was found to be stable for at least one hour after the initial 30-minute period. In order to eliminate interference resulting from air bubbles,

the cuvettes[a] were rinsed with glacial acetic acid prior to the initial determination. Rinsing was not necessary afterwards.

For the preparation of the standard curve, 5 ml of a standard cholesterol solution consisting of 0.4 mg of cholesterol (recrystallized four times from alcohol) and 0.3 ml of 33 per cent KOH (5 g of reagent grade KOH dissolved in 10.0 ml of distilled water) were thoroughly mixed. These were then saponified in a water bath at 37-40° for 55 minutes and extracted concurrently with the total cholesterol samples. Aliquots consisting of 0.10, 0.25, 0.50, 0.75, 1.0 and 2.0 ml were removed consecutively from the petroleum-ether layer and used for the standard curve. These represented quantities of cholesterol of 0.02 mg, 0.05 mg, 0.1 mg, 0.15 mg, 0.2 mg and 0.4 mg. A standard curve was prepared for each experiment.

Because the development of color as well as its stability are dependent on numerous factors, a preliminary study was performed to determine the optimal conditions for the spectrophotometric determinations. Different dilutions of the working color reagent and concentrated sulfuric acid were tried in order to estimate the right proportions of each such that the color produced was within the concentration

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[a] Standard silica 1 mm cells. Beckman Instruments Inc., Fullerton, California.

range of both total cholesterol and esterified cholesterol. It was found that a ratio of 6.0 ml of working color reagent to 4.5 ml of concentrated sulfuric acid provided an excellent proportion for our purpose. Table 4 relates the various absorbancies observed with different ratios of reagents.

Table 4

Comparison of Absorbance for Cholesterol using Differing Dilutions of Reagents

Cholesterol (mg)	4 ml working reagent 3 ml conc. H_2SO_4[a]	6 ml working reagent 4.5 ml conc. H_2SO_4[a]
0.1	0.32	0.21
0.2	0.69	0.43
0.3	1.10	0.67
0.4	1.40	0.90

[a] Absorbancy at 560 mμ after 30 minutes.

The color stability was also studied by observing the absorbancies within 10 minute intervals of time. Table 5 demonstrates that the color produced is essentially stable for at least 2 hours. It was therefore decided to begin sample readings after 30 minutes and readings were completed within one hour after beginning.

4. Determination of Cholesterol Extraction Efficiency and Specificity

Because of the complexity of the extraction procedure, it was necessary to evaluate the following parameters:

i. positively identify the separated compound in the various extracts as cholesterol

ii. determine if any remaining 1-^{14}C-acetate was a contaminant in the petroleum-ether extracts

iii. determine if a uniform distribution of cholesterol in the various portions of the same liver can be ascertained

iv. determine the cross contamination of free cholesterol in the esterified cholesterol extract

v. determine the reproducibility as well as the yield of each extraction procedure.

Table 5

Stability of Absorbancy Reading as a Function of Elapsed Time

Time (minutes)	Absorbancy	Time (minutes)	Absorbancy
15	0.580	75	0.578
25	0.580	85	0.570
35	0.580	95	0.570
45	0.580	105	0.570
55	0.579	115	0.565
65	0.579	125	0.565

For these reasons the following experiments were performed:

a. Identification of Cholesterol in the Petroleum-Ether Extract

(1) Autoradiography

A thin-layer Silica Gel G. (250 microns thick)

plate was spotted with 50 µl of the unknown petroleum-ether extract, 50 µl of known ^{3}H-Cholesterol and 50 µl of 1-^{14}C-acetate (44.4 mCi/mM) and developed in a solvent system consisting of cyclohexane, ethylacetate and ethanol in the proportions of 45:45:10 respectively. When the solvent front had moved 10 cm the plates were removed from the developing tank and allowed to dry in air. The plates were then exposed to Kodak No-Screen Medical X-Ray Film[a] for a period of three weeks and an autoradiogram was produced. The film was developed in Kodak Rapid X-Ray Developer[a] for four minutes, rinsed in distilled water for 30 seconds, placed in Kodak Liquid X-Ray Fixer and Replenisher[a] for ten minutes and finally rinsed in water for twenty minutes before drying. All procedures were carried out using solutions between 19-21°. It was found that the unknown petroleum-ether extract contained only one spot and it corresponded to the known ^{3}H-Cholesterol spot. There was no spot in the unknown corresponding to the ^{14}C-acetate. It was thus decided that the extracted activity consisted only of biosynthesized ^{14}C-Cholesterol.

(2) Infrared Spectrophotometry

An infrared spectrum was determined for a

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[a] Canadian Kodak Co. Ltd., Toronto, Canada.

10 per cent solution of cholesterol in chloroform. The combined petroleum-ether extracts which contained the suspected cholesterol (taken from three rats) was evaporated and the residue reconstituted in chloroform. An infrared spectrum was then produced and the latter was found to be identical to that obtained from a known cholesterol solution.

b. Distribution of Cholesterol in Various Liver Sections of the Rat

An experiment was performed to determine if the distribution of synthesized liver cholesterol in the various sections of the same rat liver was uniform.

Three animals were injected with 10 µCi of 1-^{14}C-sodium acetate and sacrified after six hours. Two separate portions of liver were removed from each rat and the extraction for total and esterified cholesterol was carried out on each section. The amount of total and esterified cholesterol as well as the radioactivity of each was estimated in each section. Table 6 demonstrates that the radioactivity and the cholesterol content in the liver portions of each rat was within normal limits of variation.

Table 6

Cholesterol Distribution in Various Rat Liver Sections

		Total Cholesterol		Esterified Cholesterol	
Rat	Liver Section	dpm/g of tissue	mg/g of tissue	dpm/g of tissue	mg/g of tissue
1	A	5070	3.05	1400	0.85
	B	4940	3.20	1725	1.02
2	A	4090	3.00	1112	0.73
	B	4315	3.15	958	0.66
3	A	6200	2.75	1350	0.48
	B	6040	2.48	1504	0.56

c. Yield Determination in the Extraction Procedure

(1) Determination of Free Cholesterol in Esterified Cholesterol Extract

An experiment was performed to confirm that no free cholesterol was extracted into the petroleum-ether layer after precipitation with digitonin. Varying amounts of cholesterol in alcohol were added to different centrifuge tubes and thoroughly mixed with 0.2 ml of ^{3}H-Cholesterol containing 394,200 dpm. Different amounts of 1 per cent digitonin in alcohol:distilled water (1:1) were then added and the extraction for esterified cholesterol carried out. The final petroleum-ether extract obtained was placed in a liquid scintillation vial, evaporated to dryness, and counted. Thus, the radioactivity in the extracts corresponds to the

amount of free cholesterol not precipitated by the digitonin solution. Table 7 shows that an average of 2.5 per cent free cholesterol is only extracted in the petroleum-ether portion containing esterified cholesterol.

Table 7

Cross Contamination of Free Cholesterol in Esterified Cholesterol Extracts

Sample Number	Amount of cholesterol (mg)	Amount of digitonin 1% (ml)	Activity extracted (dpm)	Per cent of activity extracted
1	0.4	1	9,106	2.3
2	0 4	2	11,225	2.8
3	0.4	3	8,992	2.3
4	0.2	1	10,434	2.6
5	0.2	2	10,457	2.6
6	0.2	3	11,360	2.9
$\overline{X}$				2.5

It was thus decided that 1.0 ml of 1 per cent digitonin solution was sufficient to precipitate any amount of free cholesterol within the range of our analytical procedure.

(2) Yield Determination of Cholesterol after Saponification

An experiment was performed to determine the reproducibility and efficiency of cholesterol extraction after the saponification of esterified cholesterol. This step was necessary since the establishment of the standard

curve for the spectrophotometric analysis of the steroid involved the saponification and extraction of known amounts of the latter compound.

To each of two centrifuge tubes were added 5.0 ml of alcohol containing 2 mg of cholesterol. The latter solution was then saponified using 0.3 ml of 33 per cent KOH solution by placing the same tubes in a water bath at 37-40^{o} for 55 minutes. Thereafter, the solution was shaken with one 10-ml portion of petroleum-ether for 30 seconds. Five milliliters of distilled water were then added and the tube shaken again for 10 seconds. The tube was then centrifuged at 1000 rpm for 10 minutes. The amount of cholesterol extracted in the petroleum-ether layer was compared to a standard solution of cholesterol in alcohol. Various aliquots were analyzed representing different amounts of cholesterol. Table 8 demonstrates that the extraction procedure is essentially quantitative and reproducible.

(3) Extraction Efficiency of Total Cholesterol and Esterified Cholesterol from Liver, Kidneys and Adrenals

Since free cholesterol and esterified cholesterol are chemically different, it was deemed necessary to study the efficiency of extraction of each. However, labelled esterified

cholesterol was not available and it was necessary to biosynthesize the latter in order to use it as a tracer.

Two animals were injected with a large dose (40 µCi) of 1-^{14}C-acetate and sacrificed six hours later. Total and esterified cholesterol were then extracted using the procedure described previously. The petroleum-ether extract obtained after the precipitation of free cholesterol with digitonin contained the labelled esterified cholesterol. Known quantities of ^{14}C-labelled free or esterified cholesterol were then added to tissue homogenates (liver, kidneys or adrenals) obtained from control animals. Extraction was effected as outlined previously, and the efficiency of each determined. Thus, correction factors were established in all our calculations. Table 9 demonstrates the results obtained in the experiment.

Table 8

Efficiency of Cholesterol Extraction after Saponification

Replicate	Sample No.	Quantity of cholesterol (mg)	Absorbance after 30 min.
1	1	0.1	0.20
	2	0.2	0.44
	3	0.3	0.66
	4	0.4	0.90
2	1	0.1	0.20
	2	0.2	0.42
	3	0.3	0.64
	4	0.4	0.84
Control	1	0.1	0.20
	2	0.2	0.42
	3	0.3	0.64
	4	0.4	0.92

Table 9

Extraction Efficiency of Total and Esterified Cholesterol in Various Organs

Organ	Replicate	Total Cholesterol Per cent extraction in organ.			Average per cent extraction	Esterified Cholesterol Per cent extraction in organ.			Average per cent extraction
		Rat 1	Rat 2	Rat 3		Rat 1	Rat 2	Rat 3	
Liver	1	80.0	90.5	90.0	87.0	66.5	61.5	61.5	63.0
	2	84.5	80.5	84.5	83.0	92.5	56.5	56.5	68.0
	3	86.0	90.0	90.0	88.0	72.0	69.0	66.0	69.0
	4	90.0	85.0	91.0	89.0	--	60.0	62.0	61.0
Total Average					87.0				65.0
Kidney	1	100.0	100.0	100.0	100.0	46.0	61.0	52.0	53.0
	2	100.0	99.0	100.0	100.0	53.0	51.0	54.0	53.0
Total Average					100.0				53.0
Adrenal	1	99.0	96.0	94.0	97.0	102.0	100.0	102.0	101.0
	2	100.5	99.0	95.0	98.0	97.0	99.0	103.0	100.0
	3	93.0	95.0	92.0	93.0	96.0	102.0	102.0	100.0
Total Average					96.0				100.0

-- indicates missing value.

G. Statistical Procedures

The statistical procedures adopted for the evaluation of results observed in the acetylation study and the investigation into the various parameters affecting cholesterol metabolism were as follows:

The mean and standard error of the mean were calculated by standard statistical procedures where,

$$\bar{x} = \frac{\Sigma x}{n}$$

$\bar{x}$ = mean
Σx = sum of all observed values
n = number of observations

$$s_{\bar{x}} = \frac{s}{\sqrt{n}}$$

$s_{\bar{x}}$ = standard error of the mean*
s = standard deviation
$\sqrt{n}$ = square root of the number of observations

The intercomparison of the various groups of data was determined by means of a simple factorial analysis of variance in order to detect any significant differences. When a significant "f" value was noted, a comparison was made for treatment effect by using Duncan's New Multiple Range Test (110). The entire calculations were performed by means of an IBM/360 computer using an n-way classification of cell replicates analysis of variance program. Because some of our collected data had uneven cell sizes, the Yates formula (111) for the estimation of the missing data was employed in order that we could obtain an interaction mean

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* The standard error of the mean was used in expressing deviations of the data for the sake of clarity. It does not imply that it approximates the population standard error.

square. This formula was as follows:

$$X = \frac{rB + tT - G}{(r-1)\ (t-1)}$$

Where r and t are the number of blocks and treatments, B and T are the totals of the observed observations in the block and treatment containing the missing unit, and G is the grand total of the observed observations.

The first missing value was approximated using the formula $(X_1 + X_2)/2$.

Where X_1 and X_2 are the means of the known values for the treatment and replicate containing any one of the missing values.

RESULTS AND DISCUSSION

A. Effects of Whole-Body Gamma Radiation on Cholesterol Metabolism

When considering the following results, it is necessary to keep in mind the difference between synthesis and mobilization as discussed in the context used here. Synthesis is indicated as activity and is the amount of cholesterol which is synthesized during the six hours between injection of the carbon-14 acetate and sacrifice of the animal. Mobilization is indicative of the pool size, and is comprised of both the newly synthesized cholesterol and that which was present in the organ prior to the day of analysis. The various results were examined on the basis of a significant interaction between groups. Although the synthesis of free cholesterol in the liver and esterified cholesterol in the adrenal did not show significant interaction, trends were noted and these are also discussed. With these differences in mind, the following observations were made.

1. Effects of 50R Whole-Body Gamma Radiation on Cholesterol Metabolism

On day 1 post-irradiation there was an increase in free cholesterol (Table 10) and a decrease in esterified cholesterol in the liver (Table 11). However, the amount of

newly synthesized cholesterol was within the normal range (Tables 12,13). The kidneys and adrenals demonstrated the same response (Tables 10,11,12,13). It would appear that there is a rapid conversion of the esterified cholesterol to the free form in the liver. Although the liver may be synthesizing increased amounts of cholesterol as reported by some investigators (1,61,64,112,113), there may be a rapid clearance of the newly synthesized steroid out of the organ (4). Because the kidney is not believed to be a major organ of synthesis, it may be acting as a resevoir or buffer organ for cholesterol in the body. The adrenals, which require free cholesterol for the synthesis of other steroids, showed this initial increase in free cholesterol, probably due to the rapid radiation-induced mobilization of the precursor.

On day 3 after irradiation, a decrease of both free and esterified cholesterol was observed and the synthesis of the latter still appeared to be normal in the liver (Tables 10,11,12,13). The kidneys showed a continued increase in free cholesterol and a decrease in the esterified form. The amount of newly synthesized free and esterified cholesterol remained normal (Tables 10,11,12,13). The adrenals reflected a decrease in esterified cholesterol, but the amounts

Table 10

Amount of Free Cholesterol in Various Organs[a,b]

Day	Treatment	Liver[c]	Kidney[c]	Adrenal[e]
1	Control	1.68±0.13	2.03±0.13	2.28±0.54
	50R	1.97±0.06	3.41±0.05	4.10±0.57
	400R	1.91±0.06	2.96±0.05	2.40±0.30
	700R	1.71±0.13	2.08±0.48	26.71±1.99
3	Control	2.33±0.09	2.37±0.17	4.10±1.70
	50R	2.11±0.08	3.39±0.26	5.09±0.65
	400R	1.94±0.03	2.94±0.09	3.12±1.23
	700R	2.77±0.21	4.35±0.16	3.65±0.48
7	Control	2.28±0.08	3.33±0.14	2.99±1.06
	50R	2.01±0.07	2.97±0.13	2.17±0.62
	400R	1.96±0.10	2.93±0.10	3.52±1.32
	700R	2.42±0.12	3.65±0.08	5.41±1.13
11	Control	2.39±0.07	3.89±0.21	2.43±0.77
	50R	2.10±0.05	2.00±0.27	0.78±0.05
	400R	2.07±0.09	3.09±0.06	1.59±0.70
	700R	2.40±0.09	2.98±0.16	3.54±0.83
20	Control	2.41±0.09	3.52±0.52	2.45±1.02
	50R	1.92±0.12	1.54±0.18	2.80±1.39
	400R	2.06±0.10	2.95±0.12	2.94±0.67
	700R	2.22±0.05[d]	3.01±0.08[d]	7.99±2.80[f]

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[a] Expressed as mg of free cholesterol per g of wet weight

[b] Results expressed as mean ± one standard error of the mean

[c] Each treatment group is composed of 6 animals

[d] This treatment group is composed of 4 animals

[e] Each treatment group is composed of 6 animals and the adrenals of each two combined

[f] This treatment group is composed of 4 animals and the adrenals of each two combined

Table 10a

Analysis of Variance for the Amount of Free Cholesterol in Rat Organs[a]

Source	Liver			Kidney			Adrenal		
	df	ms	f	df	ms	f	df	ms	f
Dose	3	0.5681	2.1098[b]	3	1.3680	0.3893[b]	3	157.6400	2.1129[b]
Day	4	0.8231	3.0564[b]	4	2.0847	0.5932[b]	4	79.6080	1.0734[b]
Dose x Day	12	0.2693	4.3259[c]	12	3.5143	12.1648[c]	12	74.1640	21.4347[c]
Error	98	0.0622		98	0.2890		39	3.4600	

[a] Results analyzed for mg/g of wet tissue

[b] Not significant

[c] $p < 0.01$

Table 11

Amount of Esterified Cholesterol in Various Organs[a,b]

Day	Treatment	Liver[c]	Kidney[c]	Adrenal[e]
1	Control	1.73±0.14	3.20±0.17	42.38±2.18
	50R	0.36±0.04	0.33±0.02	18.92±1.03
	400R	0.96±0.05	1.50±0.06	26.90±3.75
	700R	0.69±0.08	2.35±0.57	24.51±0.95
3	Control	1.25±0.06	2.34±0.08	39.47±0.11
	50R	0.45±0.02	0.66±0.15	21.88±0.40
	400R	1.08±0.06	1.44±0.03	29.49±0.78
	700R	0.39±0.03	0.62±0.04	9.36±0.54
7	Control	0.34±0.04	0.56±0.05	23.26±4.41
	50R	0.46±0.04	0.83±0.17	16.35±2.60
	400R	0.97±0.04	1.44±0.07	27.34±5.06
	700R	0.35±0.02	0.51±0.03	23.79±4.64
11	Control	0.26±0.04	0.47±0.04	17.56±4.25
	50R	0.42±0.04	1.93±0.18	30.51±0.42
	400R	0.92±0.04	1.29±0.06	28.57±1.35
	700R	0.72±0.05	1.41±0.08	18.05±2.15
20	Control	0.35±0.02	1.26±0.66	19.38±2.28
	50R	0.75±0.16	3.15±0.60	28.44±1.93
	400R	0.96±0.10	1.48±0.11	31.20±7.47
	700R	1.17±0.06[d]	2.55±0.10[d]	44.42±9.97[f]

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[a] Expressed as mg esterified cholesterol per g of wet weight

[b] Results expressed as mean ± one standard error of the mean

[c] Each treatment group is composed of 6 animals

[d] This treatment group is composed of 4 animals

[e] Each treatment group is composed of 6 animals and the adrenals of each two combined

[f] This treatment group is composed of 4 animals and the adrenals of each two combined

Table 11a

Analysis of Variance for the Amount of Esterified Cholesterol in Rat Organs^{a}

Source	Liver			Kidney			Adrenal		
	df	ms	f	df	ms	f	df	ms	f
Dose	3	1.3054	1.4702^{b}	3	0.1806	0.0352^{b}	3	122.3800	0.4093^{b}
Day	4	0.6796	0.7657^{b}	4	5.8055	1.1323^{b}	4	138.5700	0.4635^{b}
Dose x Day	12	0.8876	30.6950^{c}	12	5.1273	21.7343^{c}	12	298.9900	9.9037^{c}
Error	98	0.0240		98	0.2359		39	30.1897	

[a] Results analyzed for mg/g of wet tissue

[b] Not significant

[c] $p < 0.01$

Table 12

Synthesis of Free Cholesterol in Various Organs[a,b]

Day	Treatment	Liver[c]	Kidney[c]	Adrenal[e]
1	Control	6824±2106	1709±383	13516±3659
	50R	9155±1891	2363±424	15156±2496
	400R	4140±1046	1575±314	8401±1409
	700R	4140± 579	1695±128	7765±1148
3	Control	5277±1376	1488±268	5952± 645
	50R	6219± 925	1635±220	6862± 533
	400R	6998± 881	1147±129	8092±1638
	700R	6982± 910	1056±145	9231± 789
7	Control	5216±1689	3624±1284	5060±1271
	50R	9427±3016	1566± 234	7834±1467
	400R	8664±2713	1269± 355	8464±2968
	700R	12511±2413	3217± 840	20313±6296
11	Control	7457±2644	1363±313	7361±2200
	50R	11155±3612	927±368	11528±2275
	400R	4762± 488	1154±132	4724± 738
	700R	8237±2049	906±205	8616±2616
20	Control	6934±1494	740±102	8288±1026
	50R	5288±1083	177± 22	5998±1301
	400R	4324± 650	678± 97	4355±1584
	700R	5316±1411[d]	1316±251[d]	8907± 117[f]

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[a] Expressed as dpm of free cholesterol per g of wet weight

[b] Results expressed as mean ± one standard error of the mean

[c] Each treatment group is composed of 6 animals

[d] This treatment group is composed of 4 animals

[e] Each treatment group is composed of 6 animals and the adrenals of each two combined

[f] This treatment group is composed of 4 animals and the adrenals of each two combined

Table 12a

Analysis of Variance for the Synthesis of Free Cholesterol in Rat Organs[a]

Source	Liver			Kidney			Adrenal		
	df	ms	f	df	ms	f	df	ms	f
Dose	3	35,751,000	1.7140[b]	3	2,349,600	1.0940[b]	3	46,231,000	1.0690[b]
Day	4	51,928,000	2.4890[b]	4	10,645,000	4.9600[c]	4	44,322,000	1.0250[b]
Dose x Day	12	26,604,000	1.2750[b]	12	2,145,900	2.0150[d]	12	43,224,000	2.7510[e]
Error	98	20,856,122		98	1,064,796		39	15,711,538	

[a] Results analyzed for dpm/g of wet tissue

[b] Not significant

[c] $p < 0.025$

[d] $p < 0.05$

[e] $p < 0.01$

Table 13

Synthesis of Esterified Cholesterol in Various Organs[a,b]

Day	Treatment	Liver[c]	Kidney[c]	Adrenal[e]
1	Control	1883±659	904±193	24946±8547
	50R	2631±662	987±138	28724±5241
	400R	1232±167	764±100	21253±2353
	700R	826± 92	675±102	18315±1301
3	Control	1355±325	1041±119	15378±4346
	50R	1167±165	1128± 89	20506±3267
	400R	1870±372	566± 32	27620±7006
	700R	906±294	266± 36	10764±1610
7	Control	1163±194	1347±186	19949±6158
	50R	1788±345	1360±131	16072±4306
	400R	1781±201	1527±141	24222±1759
	700R	3042±752	1226±268	43122±17530
11	Control	1188±193	1288±114	23255±828
	50R	2408±823	2743±467	42546±10220
	400R	1418±148	1599± 64	19891±2707
	700R	1822±327	1522±103	15732±1604
20	Control	1403±339	1603±364	17503±6219
	50R	1046±322	1624±140	14778±3844
	400R	1544±235	1526± 62	18580±6896
	700R	1505±383[d]	1214±172[d]	15795±8217[f]

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[a] Expressed as dpm of esterified cholesterol per g of wet weight

[b] Results expressed as mean± one standard error of the mean

[c] Each treatment group is composed of 6 animals

[d] This treatment group is composed of 4 animals

[e] Each treatment group is composed of 6 animals and the adrenals of each two combined

[f] Each treatment group is composed of 4 animals and the adrenals of each two combined

Table 13a

Analysis of Variance for the Synthesis of Esterified Cholesterol in Rat Organs[a]

Source	Liver			Kidney			Adrenal		
	df	ms	f	df	ms	f	df	ms	f
Dose	3	846,810	0.3520^{b}	3	1,893,800	3.4310^{b}	3	56,816,000	0.3960^{b}
Day	4	1,628,500	0.6770^{b}	4	4,631,900	8.3930^{d}	4	205,690,000	1.4360^{b}
Dose x Day	12	2,402,700	2.4480^{c}	12	551,830	2.6400^{c}	12	256,010,000	1.7880^{b}
Error	98	981,378		98	208,949		39	143,164,102	

[a] Results analyzed for dpm/g of wet tissue

[b] Not significant

[c] $p < 0.01$ level

[d] $p < 0.005$ level

of free cholesterol and synthesized free steroid were normal. The synthesis of esterified cholesterol also appeared normal (Tables 10,11,12,13). The liver may have been acting as a regulator (114) and continued to mobilize free cholesterol although it contained less than normal quantities. In other terms, the demand in the body was greater than the available supply. Although the liver supply of free cholesterol was being exhausted, the kidneys seemed to retain some of their reserve pool. On the other hand, the adrenals appeared to have a normal amount of free cholesterol which would suggest that there has been a rapid conversion of the latter into other steroids until day 3 and now the available amount of free cholesterol is equal to that required for the synthesis of these steroids.

The liver showed a continued decrease in free cholesterol on day 7 although the amount of esterified cholesterol and its synthesis had increased slightly (Tables 10,11,12,13). The kidneys exhibited a decrease in free cholesterol along with its synthesis and an increase in the amount of esterified cholesterol even though the synthesis was normal (Tables 10,11,12,13). In the adrenals, the amount of free cholesterol was normal whereas the amount of newly synthesized had increased. The amount of esterified

cholesterol was below normal and the amount of the newly synthesized ester still appeared normal (Tables 10,11,12,13). It seems that, through some unknown mechanism, the liver sensed the depletion of free and esterified cholesterol and thus began synthesis of cholesterol esters first. The kidney had also depleted its reserve supply of free cholesterol and its newly synthesized cholesterol was being used for the manufacture of other steroids. This is shown in the adrenal where an increase in synthesized cholesterol was observed although the pool appeared to be normal. We also noticed an increase in specific activity (dpm/mg of free cholesterol), thus confirming our previous hypothesis.

On day 11, there was a continued increase in the pool and synthesis of esterified cholesterol in the liver. The amount of free cholesterol was still below controls and its synthesis appeared normal (Tables 10,11,12,13). The kidneys and adrenals also reflected these conditions (Tables 10,11,12,13). The liver exhibited the same results as on day 7 with the exception that the ratio of esterified to free cholesterol had increased, thus indicating that the liver may have over-compensated in its synthesis of cholesterol ester. The kidneys showed the same results but it appeared

the majority of the esterified cholesterol, being formed and/or mobilized, had done so prior to day 7, as the specific activity (dpm/mg of esterified cholesterol) was decreased. Similar results were also noted in the adrenals. Although there appeared to be an increase in the amount of newly synthesized esterified cholesterol, the amount from the pool was also significant as the specific activity was not increased.

The liver showed a continued decrease in free cholesterol and an increase in esterified cholesterol on day 20. However, the amount of newly synthesized esterified cholesterol was normal and the synthesis of the free steroid also showed this trend (Tables 10,11,12,13). There was also a decrease in free cholesterol and an increase in esterified cholesterol in the kidneys. The amount of synthesized cholesterol esters was normal but the synthesis of free cholesterol seemed to be depressed (Tables 10,11,12,13). In the adrenals, there was an apparent elevation of cholesterol esters but the free cholesterol and its synthesis was normal. The synthesis of esterified cholesterol also appeared normal. (Tables 10,11,12,13). The rate of synthesis in the liver seemed to be the same as that of control levels by day 20 although the pool size was still disturbed. It appeared, therefore, that the general pool takes a longer time to

equilibrate than the mechanism for synthesis in this organ. The kidneys showed results similar to those of the liver except that there was a decrease in free cholesterol synthesis. The latter was probably being transported to some organ other than the adrenals. This was confirmed by the fact that the adrenals did not show an increase in free cholesterol activity. However, the adrenals showed increased amounts of esterified cholesterol, possibly due to over-compensation.

It would appear that 50R of ionizing radiation caused an initial increase in the mobilization of the cholesterol pool with a rapid conversion of esterified to free cholesterol. By day 3 the kidney appeared to replace the liver in supplying the demand of free cholesterol required by the adrenal. This continued through day 7 where the kidney also transported more synthesized cholesterol to the adrenals. The body attempted to replenish the depleted pool of esterified cholesterol before the free pool and may have initially over-compensated in doing so. Even by day 20 post-irradiation, the pool of free and esterified cholesterol had not returned to normal. Whole-body irradiation with 50R did not appear to have any effect on cholesterol synthesis, and the rate of synthesis was changed after 7 days in order to replenish the cholesterol pools in the body.

2. Effects of 400R Whole-Body Gamma Radiation on Cholesterol Metabolism

On day 1 post-irradiation, free cholesterol increased and esterified cholesterol decreased in the liver. The rate of synthesis of esterified cholesterol was not affected and the synthesis of the free steroid also appeared to be normal (Tables 10,11,12,13). This was also noted in the kidneys and adrenals except that in the adrenals, the amount of free cholesterol was similar to control levels (Tables 10,11,12,13). It appeared that both the liver and kidneys rapidly mobilized cholesterol and the esterified form was converted into free. The adrenals showed the same results except for the amount of free cholesterol, and this would suggest that the free cholesterol, present in this organ, was rapidly being converted into other steroids. The synthesis of both free and esterified cholesterol did not appear to be affected by this dosage.

On the third day after irradiation, the liver exhibited a decrease in the free and esterified cholesterol. The synthesis of esterified cholesterol was normal and the free cholesterol synthesis also showed this trend (Tables 10, 11,12,13). The amount of free cholesterol was still above control values in the kidneys although its synthesis remained

normal. Both the pool and synthesis of the esterified cholesterol were below control values (Tables 10,11,12,13). In the adrenals, the amount of free cholesterol and its synthesis were normal. However, the esterified cholesterol pool was low and the amount of synthesized ester appeared elevated (Tables 10,11,12,13). The liver appeared to be depleted of its initial resevoir of free cholesterol and the kidney, acting as a reserve, still had an excess of free cholesterol. However, there was a reduction in the amount of synthesized cholesterol esters and it seemed that these were transported to the adrenals as this organ showed an increase in esterified cholesterol activity and the specific activity of the esterified cholesterol pool was increased. The free cholesterol pool and the rate of synthesis were still normal suggesting that the adrenals were synthesizing steroids as rapidly as required.

On day 7 post-irradiation, the free cholesterol in the liver was still below control values and its synthesis still appeared normal. However, the amount of esterified cholesterol had increased as well as its rate of synthesis (Tables 10,11,12,13). In the kidneys, we observed a decrease in the free cholesterol pool and a decrease in its rate of synthesis. The quantity of esterified cholesterol remained

elevated but its rate of synthesis had returned to normal (Tables 10,11,12,13). The adrenals appeared to have returned to normal function (Tables 10,11,12,13). The liver again exhibited stimulated synthesis of esterified cholesterol to replenish the pool which was depleted during conversion to free cholesterol. However, it seemed to have overcompensated as the esterified cholesterol pool had now increased. The specific activity of the esterified cholesterol pool was decreased and this suggested that the majority of the esterified cholesterol was available before day 7. Although we observed a decrease in the free cholesterol pool in the kidneys, we also noticed that the rate of synthesis was lower than normal. Since the amount of esterified steroid had increased, and its specific activity had decreased, it is suggested that the conversion of the free to the ester form took place prior to day 7. It is interesting to note that the adrenals seemed to maintain a precarious balance between the mobilization and the rate of synthesis of free and esterified cholesterol on this recovery day.

On day 11 post-irradiation, the liver continued to exhibit a decrease in free cholesterol with an apparent normal rate of synthesis. There was an increase in esterified cholesterol and also a normal rate of synthesis of the latter

(Tables 10,11,12,13). In the kidneys there was a decrease in free cholesterol although its synthesis was normal. Both the esterified cholesterol and its rate of synthesis were elevated (Tables 10,11,12,13). The adrenals were normal except for an elevation in the esterified cholesterol pool. The synthesis of esterified cholesterol also appeared normal (Tables 10,11,12,13). The rate of synthesis of esterified cholesterol in the liver had returned to normal but there was a continued increase in the amount of esterified cholesterol indicating that synthesis had continued to occur after day 7. The kidneys had an increased rate of synthesis of esterified cholesterol and because there was a decrease in specific activity, it would indicate that this ester was either rapidly transported out of the organ or that the majority had been synthesized before day 11. The adrenals appeared normal except for an increase in the amount of esterified cholesterol which suggested that there might have been an overcompensation.

By day 20, the liver still exhibited a low level of free cholesterol in the pool and its synthesis also appeared to be decreased. The esterified cholesterol pool remained elevated although its rate of synthesis was normal (Tables 10,11,12,13). The kidneys contained normal amounts

of both free and esterified cholesterol in the pools and their synthesis was also normal (Tables 10,11,12,13). In the adrenals, the free cholesterol was at a normal level but there was a decrease in synthesis. The esterified cholesterol pool was elevated but its rate of synthesis was similar to that of the control (Tables 10,11,12,13). The trend of free and esterified cholesterol was not much different from day 11 post irradiation. Because the specific activity of the esterified cholesterol was decreased while no change was observed in the rate of synthesis, it is suggested the increase in the ester pool resulted prior to day 20. The kidney appeared to be normal as far as synthesis and pool content of both free and ester cholesterol. It may be that esterified cholesterol was moving into the kidneys from other sources which leads to a decrease in specific activity. A lower synthesis rate of free cholesterol than controls was observed in the adrenals. Because the pool was normal, it is implied that they replenished their content of free cholesterol from other sources in order to maintain this normal level. On the other hand, the ester which had shown a decrease in specific activity while maintaining a normal rate of synthesis and an increased pool size must have accumulated the excess amount prior to day 20.

The initial effects of 400R gamma radiation appeared to be like those after 50R in that they caused a rapid mobilization of the cholesterol along with a rapid conversion of esterified to free cholesterol. The kidneys seemed to replace the liver in supplying free cholesterol by day 3 post-irradiation. After seven days the animal appeared to have recovered and began to replenish the depleted cholesterol ester pools and once again it seemed to overcompensate in doing so. It is suggested that the rate of synthesis was not affected by 400R of gamma radiation and that the increases noted were the result of a feedback mechanism to replenish free and esterified cholesterol which were changed during the initial mobilization. Although we showed no initial effect on the synthesis of cholesterol after exposure to 400R, some investigators have reported otherwise. Hanel *et al.* (2) found that there was a decreased incorporation of 2-^{14}C-acetate into liver cholesterol during the first day after exposure to this dose, and Vinogradova (63) has shown that there was an increased rate of renewal of cholesterol in the first 24 hours.

3. Effects of 700R Whole-Body Gamma Radiation on Cholesterol Metabolism

On day 1 post-irradiation we observed an apparent

decrease in the synthesis of free cholesterol although the pool was the same as that of the control. The amount of esterified cholesterol was decreased as well as its synthesis (Tables 10,11,12,13). In the kidneys, the free cholesterol pool and synthesis were both similar to the controls. The synthesis of cholesterol esters was also the same as normal but the ester pool was decreased (Tables 10,11,12,13). The adrenals exhibited an increase in the free cholesterol pool but the symthesis was decreased. There was also a decrease in the ester pool but its synthesis seemed normal (Tables 10, 11,12,13). The esterified cholesterol pool had rapidly been depleted in the liver and possibly converted to the free form. However, it seemed that the mechanism for the synthesis of esterified cholesterol had been inhibited. The supply of free cholesterol was at the same level as the controls but its synthesis also appeared to be slightly depressed. It is also possible that it may be rapidly transported from the organ but it was impossible to determine this from our study. In the kidneys, the only change was the depletion of the ester pool which might have been converted to the free form and this also rapidly transported away. The adrenal was the only organ to show an initial increase in free cholesterol. It had probably been transported from

the liver and kidneys at a faster rate than in the case of other irradiation dosages. The majority of this free cholesterol was transported early as indicated by the decrease in specific activity. It is uncertain whether the decrease in the synthesis of free cholesterol was due to the effects of irradiation or to the excess amount present. The esterified cholesterol had probably been converted to the free form in the adrenal also.

On day 3 after irradiation there was an increase in free cholesterol with an apparent normal rate of synthesis in the liver. The cholesterol ester pool was decreased but its synthesis was normal (Tables 10,11,12,13). The free cholesterol pool was also elevated in the kidney but its synthesis was depressed. The esterified cholesterol pool was also low as well as its synthesis (Tables 10,11,12,13). The adrenals showed an increase in free cholesterol synthesis but the pool was normal. There was a decrease in the ester pool but its synthesis appeared normal (Tables 10,11,12,13). Thus, there seemed to have been a continued mobilization in the liver and the supply of free cholesterol had exceeded the demand resulting in an excess present. The synthesis had returned to normal but the ester pool was still low. The same effect was noted in the kidney although its synthesis

of free and esterified cholesterol was now depressed. It is possible that this depression was due to the excess amount of free cholesterol present and not because of a direct effect of irradiation. The sythesis of esterified cholesterol may be affected by irradiation. The free cholesterol pool had returned to normal in the adrenal which would suggest there had been a rapid synthesis of steroids other than cholesterol from day 1. There was a small increase in the synthesis of free cholesterol but this is not to be considered major in nature as the specific activity was not different than that of the control. The esterified cholesterol pool was still low in the adrenals suggesting that a mobilization from the ester to the free form was still taking place in this organ.

On day 7 after irradiation, the liver showed an increase in synthesis of esterified cholesterol although the pool of each was normal. There was also an apparent increase in synthesis of free cholesterol (Tables 10,11,12,13). In the kidneys, there was an increase in the free cholesterol pool but its synthesis and the esterified cholesterol pool and its synthesis were normal (Tables 10,11,12,13). There was an increase in the free cholesterol pool and its synthesis in the adrenals. The esterified cholesterol pool was normal and its synthesis also gave values similar to those of the controls (Tables 10,11,12,13). The liver pool was

maintained at a normal level in both the free and esterified form but this was due to synthesis and not mobilization as indicated by the increase in specific activity of both. The increase in the free cholesterol in the kidney suggested that its reserve supply had not yet been depleted while all other parameters were normal. The increase in free cholesterol which was observed in the adrenal glands was newly synthesized as shown by the increase in free cholesterol activity and also an increase in specific activity.

On day 11, the free cholesterol pool remained normal in the liver and its synthesis indicated a return to a normal state. However, the esterified cholesterol pool was now elevated as well as its synthesis (Tables 10,11,12,13). In the kidneys, the free cholesterol pool was reduced although its synthesis remained normal, and both the esterified cholesterol pool and its synthesis were increased (Tables 10,11, 12,13). The adrenals showed normal levels except for the esterified cholesterol synthesis which appeared to be depressed (Tables 10,11,12,13). The liver seemed to have overcompensated on its synthesis of esterified cholesterol, and although there was an increase in the synthesis and the pool, the majority of the ester was present previous to day 11 as the specific activity was decreased. The kidneys also

showed an increase in the esterified cholesterol pool as well as its synthesis but, as in the previous case, there was a decrease in specific activity, thus indicating that the majority of the ester detected was accumulated before day 11. The depletion of the free cholesterol pool indicated it was being converted into the ester form or transferred to the adrenals and other organs. Although a depletion occurred, the synthesis remained normal. The adrenals seemed to be functioning normally and the depression in esterified cholesterol synthesis suggested that there was a slight excess of esterified cholesterol in the pool.

Twenty days after irradiation with 700R, the liver showed a depression in the free cholesterol pool although its rate of synthesis appeared to be normal. An increase in the esterified cholesterol pool was still observed while its synthesis was normal (Tables 10,11,12,13). The free cholesterol pool in the kidneys had returned to normal and there was an increase in the synthesis of free cholesterol. The esterified cholesterol pool had continued to increase but its synthesis had returned to normal (Tables 10,11,12,13). In the adrenals, the free and esterified cholesterol pools were above normal but the synthesis of the free steroid was normal. The synthesis of esterified

cholesterol also appeared to be similar to the controls (Tables 10,11,12,13). By day 20 the liver seemed to have further overcompensated in its synthesis of esterified cholesterol which might have come from the available free cholesterol in the organ. The fact that the specific activity of esterified cholesterol had decreased supports the hypothesis that this took place before day 20. There was an increase in the synthesis of free cholesterol in the kidney, possibly to replenish the depleted free cholesterol pool in the liver, and the synthesis of esterified cholesterol had returned to normal after the esterified cholesterol pool had increased. There was an increase in the esterified cholesterol pool in the adrenal which might be due to overcompensation. Because of the decrease in specific activity,it is suggested that the replenishment of the pool took place before day 20. However, the free cholesterol pool also showed an increase although its synthesis at day 20 was normal. It is possible that this excess was from the liver which showed a corresponding decrease. It may also be the result of another post-irradiation stress which required the synthesis of more adrenal steroids.

It would thus appear that the initial effect of 700R whole-body irradiation was again the stimulation of free cholesterol formation from esterified cholesterol. Such a

stimulation must have occurred very rapidly after irradiation because after one post-irradiation day, synthesis of each was depressed. This depression was also shown by Patt *et al*. (115). It is possible that this dosage inhibited the synthesis of cholesterol although Gould *et al*. (1) have found an increase in cholesterol synthesis in the liver and adrenals during the first 48 hours after exposure to 700R. The increase in free cholesterol was once again apparent in the adrenals and the liver seemed to provide a fast supply of the free steroid. Therefore, the kidneys, which may be acting as a reserve organ, continued to have an excess in free cholesterol until day 7. An excess in esterified cholesterol was not noted until day 11 which might be a sign of recovery and this was later than evidenced by the other dosage levels. The animals did not appear to become fully recovered within the time period of this study as emphasized by the constant fluctuation of the adrenal contents which might be considered as reacting to stress. This fluctuation is characteristic of a dying animal and Bacq and Alexander (85) have considered these as first and second reactions.

B. Effects of Whole-Body Gamma Radiation on Acetylation in the Liver

The effects of various doses of ionizing radiation

are summarized in Table 14.

Table 14

Effect of Whole-Body Gamma Radiation on Acetylation in the Liver[a,b]

Day after Treatment	Control[c]	50R[c]	400R[c]	700R[c]
1	20.2±1.92	11.3±1.92	16.3±0.90	19.2±1.02
3	17.9±1.71	16.6±1.18	26.5±1.43	16.8±0.53
7	15.4±1.67	19.7±2.24	17.5±2.20	17.8±2.20
11	21.8±2.04	16.4±2.04	13.7±0.53	18.9±1.14
20	12.6±0.82	13.2±1.55	19.2±1.47	13.1±1.95[d]

[a] Expressed as per cent acetylation
[b] Results expressed as mean ± one standard error of the mean
[c] Each treatment group is composed of 6 animals
[d] This treatment group is composed of 4 animals

After 50R irradiation, the ability of the liver to acetylate sulfisoxazole was reduced on the first post-irradiation day. Control levels were reached by day 3 and became slightly elevated by day 7. A reduction was observed on day 11, only to return to normal on day 20. It is interesting to note that there was no effect on cholesterol synthesis on day 1 or 3. However, there was an elevation of esterified cholesterol synthesis by day 7 which continued till day 11 but returned to normal by day 20.

Following 400R whole-body irradiation, acetylation was found to be depressed on day 1, elevated by day 3, and

to return to control levels by day 7. On day 11 post-irradiation, another depression was noted and by day 20 it was above normal. On the other hand, the synthesis of cholesterol was unaffected until day 7 when an increase in esterified cholesterol was noted, followed by normal levels on day 11 and then a depression of steroid synthesis on day 20.

After exposure to 700R there was no change in acetylation noted on any of the post-irradiation days of this investigation. However, there was an apparent effect on free and esterified cholesterol synthesis.

From these results, it was felt that no correlation could be established between the effects of irradiation on cholesterol synthesis and the acetylation step. Coenzyme A is an important factor in the formation of the steroid, and since it is reported that the rate limiting step in cholesterol synthesis occurs during the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonate (114), it was deemed necessary to study the relationship between the effect on the coenzyme and the actual synthesis of cholesterol. Because such a relationship could not be established from the resultant data, it is quite possible that other intermediates played a controlling role on the regulation of synthesis. Hanel and Wilian-Ulrich (5) suggested that irradiation did not affect the

molecule of CoA, but introduced a shift in the metabolic pathways of fatty acids and cholesterol which seems to confirm our previous hypothesis. The above authors also reported that the peak depression in the acetylation process occurred on the seventh day after irradiation with 450R. Because the animals used in the latter investigation were older (7-8 weeks old) in comparison to those used in this study (3-4 weeks old), it thus seemed that there was an age dependency as far as the effects of radiations are concerned.

SUMMARY AND CONCLUSIONS

A. The following modes of mobilization and synthesis of cholesterol seem to derive from the above results.

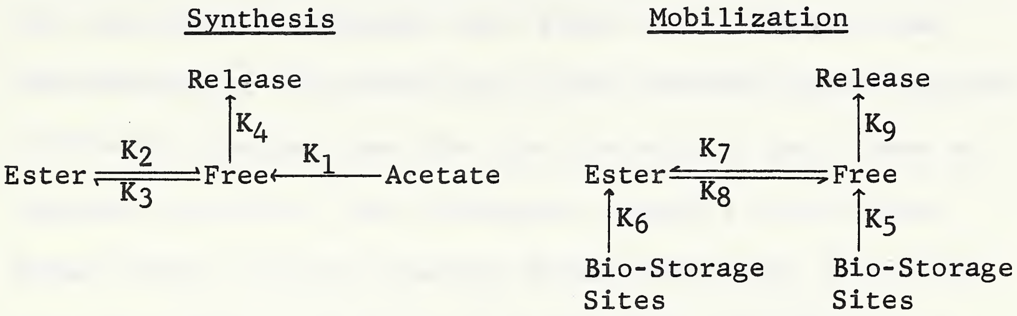

Figure 7

Scheme for the Mobilization and Synthesis of Free and Esterified Cholesterol.

It would appear that the initial effect of whole-body irradiation is one of mobilization where K_8 and K_5 are increased. Our results indicate that the synthesis process is not affected at this early stage. Then, it would appear that the value of K_9 increases in the liver resulting in a decrease of free cholesterol in the organ. This is also noted in the kidney,in which case the above events take place later than in the liver, thus suggesting that the former may be acting as a reserve supply of cholesterol. Eventually, the pool of esterified cholesterol is depleted to such an extent that K_7 and K_6 become very large in order to replenish it. An increase in the synthesis of esterified cholesterol

is now noted which implies that K_3 has also increased. It is also apparent that the body seems to replenish its esterified cholesterol pool first and in doing so may overcompensate, thus resulting in the observed excess present. It is also observed that the free cholesterol pool takes an extensive period of time to regain normality in the liver. Normal levels are not reached during the 20-day period of each experiment. It does not appear that radiation has a direct effect on the synthesis of cholesterol, but the stimulation or depression seems to be regulated by the amount of the steroid present in the pool. Although exposure to 700R of ionizing radiation appears to cause a depression of cholesterol synthesis on day 1 post-irradiation, it is not possible to conclude within the scope of our experiment, if this is a direct effect. Noteworthy is the fact that under all irradiation dosages, the ester pool is replenished prior to day 20 as evidenced by a decrease in specific activity of the ester in all organs examined.

B. A significant main treatment effect observed is a day effect on the synthesis of both free and esterified cholesterol in the kidney. Table 15 summarizes these observations. Those values not underscored by the same line are significantly different. It can be seen that a direct relationship exists

between the synthesis of the cholesterol esters and the time after irradiation with a maximum increase on day 11. It is also observed that the synthesis of free cholesterol is greatest during the first few days post-irradiation with a maximum at day 7.

Table 15

Radioactivity of Free and Esterified Cholesterol at Various Post-Irradiation Times in the Rat Kidney[a]

	Free Cholesterol (dpm/g of organ)					Esterified Cholesterol (dpm/g of organ)				
day post-irradiation	20	11	3	1	7	3	1	7	20	11
activity	712	1088	1332	1836	2419	750	832	1365	1463	1788

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[a] Subsets are significant at the 5 per cent level.

C. There seems to be no correlation between the rate of acetylation after irradiation, and the actual rate of cholesterol synthesis.

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